STUDIES ON ALKALINE PROTEASES OF

Spodoptera litura

By

ZAHEER AHMAD

Department of Chemistry
Biochemistry Division
Aligarh Muslim University
Aligarh

SUMMARY

A Thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science of Aligarh Muslim University, Aligarh.

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SUMARY

The alkaline protease activity in the gut of Spodoptera litura was found to increase with the development of larvae and decreased with the onset of pupation. The pattern of changes in protein concentration in the gut was parallel to the protease activity. During day 7 to day 9, the larvae became more voracious feeder which presumably accounts for the enhanced protein concentration in the gut. The protein concentration falls after day 9 as the larvae gradually become pupae and give up food consumption.

The change in protease activity during starvation of the larvae showed an abrupt increase at 4 hours which decline consistently on further starvation. There was a slight decrease in protein concentration of gut content at 4 hours with a slower decline on further starvation.

Incubation of crude enzyme solution at 37 ± 1°C for 55 hours resulted in a sharp decrease in protein concentration until 22 hours without any further significant change until 55 hours. The enzyme activity remained almost unaltered till 22 hours but decreased subsequently till 55 hours. A 42% loss in enzyme activity was observed when the crude enzyme solution was dialysed at 37°C, whereas only 10% loss in activity was observed at 4°C.

To purify the alkaline proteases, the fifth instar larvae of Spodoptera litura were dissected and their intestines collected in an ice cold beaker. The contents were squashed out by means of a glass rod in 0.1M Tris buffer, pH 8.0, to get the crude enzyme solution. Since the crude extract contains dark brown pigments and phenols, it was removed by acetone fractionation. The acetone fraction was passed through Sephadex G-75 followed by exchange chromatography on DEAE-Sephadex A-50. The alkaline proteases appeared in three well defined peaks which were homogeneous as judged by polyacrylamide gel electrophoresis. The percentage of recovery for protease I - III were 18.7, 19.4 and 13.6 with 9, 7 and 8 fold purification. Protease I - III exhibited molecular weight values of 17000, 21000 and 53000 by gel filtration on Sephadex G-200 and 18000, 23000 and 50000 by sodium dodecylsulfate polyacrylamide gel electrophoresis, respectively. The non-appearance of more than one band on SDS polyacrylamide gel electrophoresis of all the three proteases rules out the possibility of native protein III to exist in dimeric or trimeric form. The Stoke's radii for protease I - III were calculated as 1.89 nm, 2.16 nm and 3.08 nm respectively. The values of diffusion constant for the three proteases were respectively 11.79×10^{-2} cm^2/Sec, 10.32×10^{-7} cm^2/Sec and 7.24×10^{-7} cm^2/Sec. These observations suggested that protease I and II are more compact and globular than protease III.

The optimum pH for the crude extract was 11.0, with a shoulder between pH 8.0 and 9.0. Optimal pH for protease I - III

using casein as substrate were 11.0, 10.5 and 9.0 respectively. The temperature optima for protease I - III obtained by 20 minutes reaction at pH 8.0 were 60°C, 55°C and 50°C respectively. The effect of pH on the stability of enzyme showed that protease I and II are relatively more stable than protease III at extremes of acidic or alkaline pH. There was no remarkable difference in terms of thermal inactivation of all the proteases. The inactivation began at 45°C, and at 60°C, almost all the proteases lost fully activity. Lineweaver-Burk plots were obtained with each of the proteases using casein as substrate. The Km values obtained were 5.7×10^{-6} M, 2.9×10^{-6} M and 2.1×10^{-6} M respectively for protease I, II, and III. All the proteases hydrolysed BAPA, BAEE but not BTEE suggesting the trypsin-like nature. Protease I is most active against casein as well as BAPA as a substrate. Though, protease III was more active than protease II against casein, the situation was reverse for BAPA as substrate.

In an attempt to understand about the nature of the enzymes, the effect of various inhibitors on protease activity was studied. Metal chelating agent, EDTA, did not show any inhibitory effect on the proteolytic activity of each enzyme. The activity of each proteases remained unaltered after the treatment with the thiol specific reagents, iodoacetic acid, N-ethylmaleimide, PCMB, β-mercaptoethanol and cysteine hydrochloride. Like trypsin and chymotrypsin all the proteases were completely inhibited by N-bromosuccinimide. PMSF which is known

to react specially with functional serine residue, inhibited all the proteases. TLCK was inhibitory to all the proteases indicating the participation of histidine in the active site. The chymotrypsin specific inhibitor, TPCK had no inhibitory effect on any of the enzymes. Each of the proteases were inhibited by SBTI and LBTI but ovomucoid only inhibited the protease I and II.

These proteases required no metal ions for their full activity. Ca^{++}, Co^{++}, Mg^{++} and Mn^{++} had almost no influence on the activity whereas the heavy metal ions like Zn^{++} and Cu^{++} caused strong inhibition. However, Hg^{++} caused complete inhibition of all the three proteases.

No evidence could be found for the existence of any of these proteases as inactive precursors.

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ZAFEER AHMAD

Department of Chemistry
Biochemistry Division
Aligarh Muslim University
Aligarh

Dated.....
Approved :

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(A. M. Siddiqui), *Supervisor*

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C E R T I F I C A T E

I certify that the work presented in this thesis has been carried out by Mr. Zafeer Ahmad and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

(A.M. Siddiqi)
Professor of Biochemistry
Department of Chemistry
Aligarh Muslim University
Aligarh - 202001 (India)

DEDICATED

TO

MY

PARENTS

A C K N O W L E D G E M E N T S

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C O N T E N T S

LIST OF ABBREVIATIONS

BAPA	α-N-Benzoyl-D, L-arginine p-nitroanilide
BAEE	N-Benzoyl-L-α-arginine ethyl ester.
BTEE	N-Benzoyl-L-α-tyrosine ethyl ester.
PMSF	Phenylmethylsulfonylfluoride.
TLCK	N-α-p-Tosyl-L-lysinechloromethyl ketone HCl.
TPCK	L-1-Tosylamide-2-phenylethylchloromethyl ketone.
SBTI	Soybean trypsin inhibitor.
LBTI	Limabean trypsin inhibitor.
pCMB	p-Chloromercuribenzoate.
SDS	Sodium dodecylsulfate.
TCA	Trichloroacetic acid.
EDTA	Ethylenediamine tetracetic acid.
nm	Nanometer.
M	Molarity
µmole(s)	Micromole or micromoles.
gm	Gram or grams.
µg	Microgram or micrograms.
hr	Hour or hours.

PREFACE

Inspite of green revolution, insects continue to be a menance for agricultural productivity. Much efforts have been made to characterize mammalian, fungal or bacterial proteases but relatively less information is available on insect proteases. A survey of literature till June, 1977 in the first chapter would indicate that most of the workers reported only the occurrence and some preliminary properties, mainly substrate specificity of the insect proteases. In only few cases detailed informations on the purification and physico-chemical properties of insect proteases are available. The main reason for our limited knowledge of insect proteases, specially of the digestive tract, is the paucity of enough raw material for detailed study. In the present investigation, we have therefore, selected *Spodoptera litura* (army worm). The insect can be reared in almost unlimited quantity at room temperature ($30 \pm 2^{\circ}$) without any difficulty. The large size of the insect is an additional advantage to get enough gut content to undertake purification of proteases. Ishaaya *et al.* (1971) have reported the occurrence and preliminary properties of a protease with an unusually alkaline pH optimum in the larvae of *Spodoptera littoralis*. However, no systematic attempts have so far been made to purify and characterize the enzyme. The present work describes the isolation, purification to homogeneity and physico-chemical properties of three proteases present in the larval gut of *Spodoptera litura*.

CHAPTER I

I N T R O D U C T I O N

Insects, which constitute an extremely large group of animal kingdom, can cope up with extremes of condition and can live "just about anywhere - and on just about anything". The broad range of habitat and extreme broad range of dietary habits make this class the most serious competitor, man could ever encounter on earth. Numerous studies have been performed on insect nutrition and special attention has been paid to carbohydrates and proteins as most species derive their nourishment from these nutrients. Since the utilization of these nutrients depends on digestive enzymes, the study on these enzymes is important. The earliest investigations on the digestive enzymes of insect have been accredited to Plateau (1875) and subsequently to Swingle (1925) and Wigglesworth (1927). The nature of the proteolytic enzymes present in the gut homogenate of insects is in general similar to that of other invertebrate animals (Day and Waterhouse, 1953). The insect proteases, like those of the vertebrates, are usually made up of several components: proteinases acting upon natural proteins, and a group of peptidases by which the products of protein digestion are further hydrolysed. In most cases, the proteinases are of trypsin-like and very rarely pepsin-like (Wigglesworth, 1972). Insects which live on food rich in some particular substance generally produce the appropriate enzymes in more abundance; and in those which live on highly restricted diet, the enzymes present are correspondingly limited.

The digestive enzymes in insects are adapted to the diet on which they feed. Stored products Tenebrionids, particularly the mealworm, Tenebrio molitor L., and the flour beetles, Tribolium confusum have long been recognised as good test animals for experiments in insect nutrition. Moreover, since the general nutritional requirements of insects are in many respect similar to those of warm-blooded animals (Hinton, 1956), these beetles have, therefore, been utilized as models for study of humans and livestock nutrition (Chirigos et al., 1960; Birk and Applebaum, 1960). A pre-requisite for the utilization of such insects for the bioassay of foodstuffs and understanding of insect nutrition requires better knowledge of their digestive enzymes. Kretovich et al. (1943), Kretovich (1944) and Nuorteva (1954) described the activity of salivary protease of Eurygaster integripes Put. and Lygus rugulipennis Propp. which varied with season and that protein diet stimulated the secretion of the protease. Nuorteva and Laurema (1960) reported that the secretion of the salivary protease of Dolycoris baccarum was also stimulated by the albumin diet. The kind of food given influenced the secretion of digestive enzymes in the polyphagous Prodenia eridania Cramer (Soo Hoo and Fraenkel, 1966) and in the oligophagus Protoparce sexta Johan (Waldbauer, 1962). Ishaaya et al. (1971) reported that the larval digestive enzymes of the Spodoptera littoralis Boisd are affected by the type of food. Protein was found to be a critical factor affecting digestive enzymes, protease as well as amylase.

Numerous attempts have been made to investigate the digestion of proteins by Tenebrionid stored-product insects (Powing et al., 1951; Lipke et al., 1954 and Dadd, 1956). Interest has to some extent been directed to the elucidation of factors affecting midgut proteolytic secretion such as food ingestion (Dadd, 1956), temperature fluctuation (Birk et al., 1962) and compensation for temperature (Applebaum et al., 1963). The SBTI, and ovomucoid inhibit Tenebrio proteases (Applebaum et al., 1964) but not the Tribolium proteases (Lipke et al., 1954 and Birk and Applebaum, 1960). However, another inhibitor found in soybean could inhibit the Tribolium proteases (Birk et al., 1963).

There are a number of studies on the presence of digestive enzymes in phytophagous bugs. Nuorteva (1954) reported that five species of Pentatomidae had amylase and proteases in their salivary gland. D. fasciatus has a relatively wide variety of digestive enzymes, lipase, peptidase, α-glucosidase, β-glucosidase and amylase, in the salivary gland (Ford, 1962; Khan & Ford, 1967). Coridius janus contains amylase, proteinase and esterase (Rastogi, 1961; Rastogi & Datta Gupta, 1962a). Odonotopus nigricornis contains amylase and proteinase (Rastogi, 1962; Rastogi & Datta Gupta, 1962b). Clavigrella gibbosa contains amylase maltase and protease (Mathur & Thakar, 1969). Hori (1970a,b,c) found amylase and protease in the salivary gland of Lygus disponsi of the family Miridae. The properties

of enzymes, in particular of amylase, protease and invertase in the digestive system of Lygus disponsi Linnavuori (Hemiptera, Miridae) a pest of legume and sugar beet, were first investigated by Hori (1973). The role of these gut enzymes in digestion was compared with those of the salivary gland. The salivary protease was usually higher in activity than the gut protease, the former on average was about 5 times more active than the latter. The salivary amylase and protease activities of L. disponsi varied with the developmental stage and markedly increased just before moulting. Takanona and Hori (1974) reported the presence of digestive enzymes in the salivary gland and midgut of Stenotus binotatus Fabricius (Hemiptera: Miridae). Inspite of the presence of protease they have reported the occurrence of various carbohydrase (trehalase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase), amylase and esterase. It was found that amylase, α-glucosidase, pectinase and protease were present both in the salivary gland and the midgut of S. binotatus, while β-glucosidase, α-galactosidase and esterase were found only in the midgut.

The properties of proteolytic enzymes of the digestive fluid and midgut of the Silkworm larvae, Bombyx mori, were investigated by Hori et al. (1963). Compared to midgut much higher activity was present in the digestive juice. The protease activity of digestive juice varied with the development of larva. Eguchi and Yoshitake (1967) reported the electrophoretic

variation and the mode of inheritance of protease in the digestive fluid. There are many studies on the cocoon-digesting enzymes of Bombyx mori (Watanabe, 1926; Akabori; Uehara, 1942 and Duspiva, 1950). Kafatos and Williams (1964), Kafatos et al. (1967a,b) and Berger et al. (1971) studied the cocoonase produced by moths of the genus Antheraea. Proteolytic enzymes in the midgut of the pharate adult of the silk worm, Bombyx mori, have been reported by Eguchi et al. (1972). Most of the proteolytic activities were detected in the contents of the midgut of the pharate adult but not in the midgut tissue. The activity increased in the pharate adult period, reached a peak just before emergence of the moth, and decreased markedly thereafter. On the basis of electrophoretic pattern of the enzyme, the authors believed that the enzyme is different from that of the cocoon-digesting enzyme. The influence of intermittant fasting on proteolytic activity of the midgut as related to the growth of larvae of Bombyx mori was studied by Janda and Maxinerova (1972). The long term intermittant fasting showed unfavourable effects on both proteolytic activity and larval development. On the other hand, short-term intermittant fasting followed by normal food intake showed positive effects on both enzyme activity of the gut and larval growth. Eguchi et al. (1974) reported the properties of the protease in the crop fluid and maxillae of the silkworm, Bombyx mori by agar-gel electrophoresis, gel filtration, and enzyme activity assay on different substrates. Protease in the late-pupal crop fluid and in the adult moth exudate hydrolysed casein, solubilized fibroin and

liq-state native silk. This enzyme had two pH optimum values of 7.7 and 9.0. When the enzyme preparation was passed through a Sephadex G-150 column, it showed two active peaks. Eguchi and Iwamoto (1976) reported the presence of alkaline proteases in the midgut tissue and digestive fluid of the silkworm, Bombyx mori. The pH-activity curves of both proteases were very similar and optimal activity was about pH 11.2. $HgCl_2$ and DFP strongly inhibited protease activity, and the influence was greater in digestive fluid than in the midgut tissue.

Insects are the only animals in which digestion of keratin has been demonstrated, the insects involved being the larvae of some thirty species of moths (Lepidoptera), the larvae of some fifteen species of beetles (Coleoptera), and several hundred species of bird lice (Mallophaga). The most important of these insect species are the larvae of the webbing clothes moth, Tineola bisselliella (Waterhouse, 1958). The process of wool digestion in these larvae has been reviewed by Waterhouse (1958) and McPhee (1965). A complex mixture of peptidases and proteinases have been found in extracts of larval digestive tract of T. bisselliella (Ward, 1975). The proteinases included metallo-proteinases, serine-proteinases (both trypsin-like and chymotrypsin-like) but no SH-proteinases or acid proteinases. Among peptidases, both aminopeptidase and carboxypeptidase were present which were not inhibited completely by reagents specific for any of the common active sites, and had different specificity requirements.

The characterization of proteolytic enzymes in Diptera consisted simply of determining pH-activity curves on general substrates. Champlain and Fisk (1956) found considerable protease activity at pH 7.9 in the gut of Stomoxys calcitrans (L.) and concluded that a trypsin-like enzyme was present. Greenberg and Paretsky (1955) had previously reported both a trypsin-like and a pepsin-like enzyme in larvae and adults of Musca domestica (L.) Lambremont et al.(1959) reported a pepsin-like protease in the larva of S. calcitrans. Fraser et al.(1961) confirmed the presence of a trypsin-like and a pepsin-like protease in the larval blow fly, Calliphora vomitoria (L.). Patterson and Fisk (1958) electrophoretically separated 3 trypsin-like enzymes from midgut homogenates of the stable fly, S. calcitrans (L.). Patel and Richards (1960) separated a trypsin-like enzyme in the midgut of M. domestica into 2 substances by electrophoresis. Pendola and Greenberg (1975) characterized the proteolytic enzymes present in specific midgut region of the larvae of Calliphora vicina by means of specific substrates, specific inhibitors and electrophoretic separation. The data obtained from the hydrolysis of the trypsin-specific substrates, BAPA and BAEE, by separate fore-midgut, and hind-midgut homogenates at pH 7.0 confirmed that trypsin-like or chymotrypsin-like enzymes were responsible for the basic proteolytic activity in each midgut segment. At least 2 such enzymes in each midgut segment were indicated by electrophoresis, and their mobilities were distinctly less than that of mammalian trypsin. Presence of trypsin-like and chymotrypsin-like enzymes was confirmed by inhibition with SBTI.

Hydrolysis of the pepsin-specific substrates, N-carbobenzoxy-L-glutamyl-L-tyrosine and N-acetyl-L-phenylalanyl diidotyrosine suggested the presence of pepsin-like enzymes which was further confirmed by inhibition with diphenyl diazomethane.

Though a number of studies have been performed on insect proteases, only recently some attempts have been made to analyse or separate the proteolytic enzymes. An analysis of this sort is pre-requisite for the elucidation of the mechanism of proteolysis in insects, and its comparison to those of other organisms. For the first time, Applebaum et al. (1964) have separated three distinct proteolytic components of Tenebrio molitor larva which were differentiated on the basis of selective inhibition by specific trypsin inhibitors: an endopeptidase - "Tenebrio trypsin" - and two exopeptidases - carboxypeptidase B and amino-tripeptidase. The exo and endopeptidase adtivities were separated by column chromatography on ECTEOLA-cellulose, Tenebrio trypsin eluting freely. The later was further purified by adsorption on CM-cellulose and subsequent gradient elution. The relative proteolytic and esterolytic activities of both bovine and Tenebrio trypsins were similar, and esterolysis of carbobenzoxy-tyrosine nitrophenyl ester was completely inhibited by "crystalline SBTI", that was in contrast to chymotrypsin.

The larva of the blowfly, Phormia regina excretes a complex of proteolytic enzymes into the culture medium during its growth period. Brookes (1961) has partially purified a

proteolytic enzyme from P. regina which had maximal activity in the pH range 7.8 - 8.3 with casein as a substrate. The activity was unaffected by PCMB, iodoacetic acid, Mg^{++}, Mn^{++} or Na^{+}. The enzyme, hydrolyzing both BAEE and BAA was inhibited by SBTI. The enzyme resembled trypsin in some of its properties but was more stable and less soluble at an acid pH than pancreatic enzyme. The proteinase excreted by Calliphora erythrocephala has been purified and characterized by Moser (1966). The proteinase was inhibited by Cu^{++} and Hg^{++} at millimolar concentrations. Cysteine, EDTA, Mg^{++} and Ca^{++} had no effect. The Km value of the esterase activity, using p-nitrophenylacetate as the substrate, was 2.0 - 4.4 mM. The proteinase resembled the mammalian proteases in some properties but differed in specificity and pH-stability and hence the term "tryptic proteinase" has been avoided.

A protease with chymotryptic properties from the larva of Vespa orientalis, has been identified by Sonneborn et al. (1969). The protease was similar to bovine α-chymotrypsin in its inhibition by TPCK, PMSF etc. and specificity for the cleavage of the oxidized insulin B-chain, but it was of much lower molecular weight (12,500). Jany et al. (1974) have confirmed the low molecular weight nature of the enzyme using ultracentrifugation, gel filtration and polyacrylamide gel electrophoresis without SDS. These authors have also purified the enzyme and determined sedimentation coefficient, diffusion coefficient, partial specific volume, frictional ratio, and degree of hydration. Hagenmaier (1971) has purified a proteinase from the larval midgut of

Vespa orientalis by exchange chromatography on DEAE-Sephadex A-50 and gel filtration on Sephadex G-75. The molecular weight was calculated to be 27,000 by gel filtration. The specificity of esterolytic activity, inhibition with trypsin inhibitors and the cleavage specificity of the oxidized insulin B-chain allowed to speak of a trypsin-like protease.

A new class of proteolytic enzyme, cocoonase, produced by silk moths during adult development, has been crystallized by Kafatos et al. (1967a,b). Cocoonase "crystals" contained 80% of the active enzyme which was found to be a single enzyme as judged by several criteria. It has a molecular weight of 25,000 and an amino acid composition very similar to that of trypsin, except for fewer half-cystine resisdues. Cocoonase was quite stable to autodigestion at neutral and mildy alkaline pH, but was rapidly deactivated at low pH. It specifically hydrolyzed esters of basic amino acids, thus showing specificity characteristic of trypsin. Diisopropyl fluorophosphate was a potent inhibitor of cocoonase, suggesting the presence of a serine in the active site. Heavy metal ions and sulfhydryl reagents had little inhibitory effect. TLCK was inhibitory, indicating the participation of histidine in the active site. Felsted et al (1973) have studied the properties of the proteolytic enzyme, cocoonase, from Antheraea mylitta which was similar to other cocoonase and bovine trypsin in molecular weight, amino acid composition, specificity and reactivity toward various substrates. However, the A. mylitta

enzyme activated chymotrypsinogen A at a rate 32 times slower than bovine trypsin, which suggested subtle and important differences in the structures of these two enzymes.

Giebel et al. (1971) have separated and characterized four fractions (A-D) with endopeptidase activity from the midgut of Apis mellifica (honey bee). By the cleavage specificity, fraction A was characterized as trypsin, B and C did not resemble any mammalian pancreatic proteases while fraction D showed a cleavage specificity similar to chymotrypsin. With the exception of fraction C all other fractions form part of the group of "serine-proteases". Knecht et al. (1974) reported the proteolytic properties of the intestinal fluid of Locusta migratoria and presented the evidence for the existence of four distinct proteolytic fractions (P_I - P_{IV}) with endopeptidase activity. Two of them, P_I and P_{IV} resembled bovine trypsin and bovine chymotrypsin respectively. Their molecular weights were found to be 17,000 and 18,200 respectively. Fraction III did not hydrolyse any of the synthetic substrates tried and P_{II} was active only with L-glutaryl-L-phenylalanine-naphthylamide. Their molecular weights were found to be 27,000 and 32,000 respectively.

CHAPTER II

M A T E R I A L S A N D M E T H O D S

MATERIALS AND METHODS

A. MATERIALS

Sephadex G-75, G-200 and dextran blue 2000 were purchased from Pharmacia, Fine Chemicals (Sweden). DEAE-Sephadex A-50 was obtained from Sigma Chemical Company, U.S.A. Vitamin-free casein was a product of Difco Laboratories, U.S.A. Enzyme substrates, inhibitors and standard proteins were obtained from Sigma Chemical Company. These included: α-N-benzoyl-D, L-arginine p-nitoanilide, N-benzoyl-L-α-arginine ethyl ester, N-benzoyl-L-α-tyrosine ethyl ester, phenylmethylsulfonylfluoride, N-α-p-tosyl-L-lysinechloromethyl ketone HCl, L-1-tosylamide-2phenyl-ethylchloromethyl ketone, soybean trypsin inhibitor, limabean trypsin inhibitor, ovomucoid, iodoacetic acid, bovine serum albumin, ovalbumin, α-chymotrypsinogen A and bovine pancreatic trypsin. Cytochrome C was obtained from Biochemicals Unit, V.P. Chest Institute, Delhi (India). Cysteine hydrochloride, N-bromosuccinimide and ethylenediamine tetra acetic acid were obtained from B.D.H., England.

The reagents used in polyacrylamide gel electrophoresis in presence and absence of SDS were: N, N, N', N', tetramethylene-diamine (Koch-light, U.K.); acrylamide, N, N'-methylenebisacryla-mide, bromophenol blue and sodium dodecylsulfate (B.D.H., England); riboflavin, dichloromethyl silane and Amido Shwartz (E. Merck, Germany); coomassie blue and 2-mercaptoethanol (Sigma Chemical

Company, U.S.A.); acetic acid and methanol (B.D.H., India).

Other chemicals used were of analytical grade. All glass double distilled water was used throughout these studies.

B. METHODS

Rearing of Insects:

Insects were reared at 30 ± 2°C in a beaker having moistened sand at the bottom. In the larval stage they were fed with castor leaves. It was necessary to clean the beakers daily to remove their faeces. Fifth instar larvae, unless otherwise mentioned, were used throughout the studies. In the pupation stage, they did not consume any diet. They were fed upon sucrose solution in the adult stage (cotton dipped in sucrose solution wrapped around a microscopic slide was kept upright in sand at the bottom of the beaker).

MEASUREMENT OF ENZYME ACTIVITY:

(a) Proteinase Activity:

Proteinase activity was determined by the casein digestion method of Kunitz (1947) with a slight modification. The reaction mixture in a total volume of 1 ml, consisting 40 µmoles glycine-

NaOH buffer, pH 9.0 and 20 to 40 µg enzyme protein was pre-incubated for 5 minutes at 40°C. 0.5 ml of 2% casein dissolved in 0.1M of same buffer was added reincubated at 40°C for 15 min. After digestion, undigested casein precipitated with 0.5 ml of 20% TCA, was removed by centrifugation. The concentration of digested protein in the supernatant was determined using Folin reagent (Lowry et al., 1951) in a Bausch & Lomb Spectronic 20 colorimeter at 660 nm. Appropriate blanks were used in all experiments. Under the experimental conditions, one enzyme unit was equivalent to one µg tyrosine formed per min.

(b) Peptidase Activity:

Using BAPA as substrate peptidase activity was measured by the method of Erlanger et al. (1961) as described by Arnon (1970). 43.5 mg of BAPA hydrochloride was dissolved in 1 ml of dimethyl-sulfoxide and the volume was adjusted to 100 ml with 0.1M Tris buffer, pH 8.0 (care must be taken that all the BAPA is dissolved prior to the addition of the buffer). The solution was kept at a temperature above 25°C. To 0.2 ml enzyme solution, 0.8 ml of distilled water was added to adjust the total volume to 1 ml. 5 ml of the substrate was added to each tube at 1-minute interval. After 25 minutes of incubation at 40°C the reaction was terminated by the addition of 1 ml of 30% (V/V) acetic acid. The quantity of the liberated p-nitroaniline was estimated spectrophotometrically at 410 nm. The control was prepared similarly except that no enzyme was added. Under the experimental conditions one enzyme

unit was equivalent to change in O.D. at 410 nm per minute.

(c) Esterase Activity:

The esterase activity was determined by the method of Schwart and Takenaka (1955) using BAEE as the substrate. The reaction mixture contained 58 µg of BAEE in 2.9 ml of 0.1M Tris buffer, pH 8.0, containing 0.05M $CaCl_2$. 0.1 ml of enzyme solution was added and the change in absorbancy at 253 nm was followed at 25°C in a Beckman DU spectrophotometer. Esterase activity was also determined by the method of Hummel (1959) using BTEE as the substrate. BTEE was prepared by dissolving 15.7 mg in 30 ml of ethanol and making up the volume to 50 ml with water. The reaction mixture contained 1.5 ml of substrate solution and 1.4 ml of Tris buffer, pH 8.0, containing 0.1M $CaCl_2$. 0.1 ml of the enzyme solution was added and the change in absorbancy at 256 nm was followed at 25°C in a Beckman DU Spectrophotometer. Under the experimental conditions, one enzyme unit was equivalent to change in absorbancy at described wavelength per minute.

Protein Determination:

Due to high concentration of phenolic substances in the crude enzyme extract, protein was estimated by the method of Khanna et al. (1969). The protein of crude extract was precipitated by mixing 1 ml of the homogenate with an equal volume of

10% (W/V) TCA. The precipitate was initially washed three times with acetone and finally three times with alcohol. The precipitate was dried and dissolved in 0.1N NaOH by heating in a boiling water bath for 30 minutes. Any suspension, if present, was centrifuged off and suitable aliquots were used for color development according to the method of Lowry et al. (1951). However, in the purified preparation, the protein was estimated directly. A suitable aliquot was diluted to 1.0 ml with water. To this was added 5 ml freshly prepared copper reagent (by mixing 0.5% $CuSo_4$ in 1% (W/V) potassium tartarate and 2% (W/V) Na_2CO_3 in 0.1N NaOH in 1:50 ratio). After incubation for 10 minutes at room temperature, 0.5 ml of diluted Folin-Ciocalteus reagent (1N) was added and instantly mixed. The colour intensity was read after 30 minutes against reagent blank in a Bausch and Lomb Spectronic 20 Colorimeter at 660 nm. The protein concentration was evaluated from the standard plot of bovine serum albumin. The column was monitored at 280 nm using Beckman DU Spectrophotometer.

COLUMN CHROMATOGRAPHY:

Sephadex Column:

Preparative column of Sephadex G-75 was prepared according to the standard procedure of Pharmacia Fine Chemicals, Sweden. A required amount of Sephadex G-75 was allowed to swell in a suitable amount of double distilled water for 5 hours in boiling

water bath and for 12 hours at room temperature (~30°C). A glass column previously cleaned with chromic acid, detergent, water and distilled water mounted vertically and glass wool plugged along with glass beads at the bottom of the column was filled one third of its length with the operating buffer. The slurry was gently poured into the column with the help of a glass rod. The column was left standing for about one hour and after the gel had settled to some extent, the stop-cock was opened and the flow rate was adjusted gradually. The column was thoroughly washed with operating buffer (3-4 times the bed volume) to avoid the shrinking of the bed height. To check the uniform packing and to determine the void volume (V_o) of the column, 0.2 percent blue dextran solution was passed through the column. The volume of blue dextran solution used was always 2 per cent (or lower) of the total bed volume of the column. The column was washed by passing at least two bed volumes of the eluant. The buffer was then removed from the gel surface and protein solution (2-3 per cent of the total bed volume) was carefully applied with the applicator to avoid disturbance of the gel surface.

DEAE-Sephadex Column:

To ensure complete swelling of the exchanger, the required amount of DEA-Sephadex A-50 (Capacity 3.5 ± 0.5 meq/gm, particle size 40-120 μ) in suitable amount of 0.02M Tris buffer, pH 8.0, was heated in a boiling water bath for two hours and allowed to stand overnight at room temperature. A thoroughly washed glass

column (2.6 X 42 cm) plugged with glass wool was mounted vertically and filled with 50 ml of 0.02M Tris buffer, pH 8.0. The slurry was poured into the column with the help of a glass rod keeping the stop-cock closed. After one hour stop-cock was opened and the flow rate was gradually increased to the desired final flow rate. The flow rate was maintained at 50 ml/hr. The column was equilibrated with the same buffer till the pH of the effluent was identical to that of the buffer. The column was connected with the gradient assembly consisting of two gradient vessels. The mixing vessel contained 0.02M Tris buffer and the second vessel contained 1M NaCl in 0.02M Tris buffer. The linear salt gradient was checked by measuring the conductance of the effluant. The salt concentration in successive fractions was calculated using the following equations:

$$C = C_2 - (C_2 - C_1)\, e^{-V/V_o} \quad \ldots\ldots\ldots\ldots \quad (1)$$

where C is the salt concentration of the protein fraction, C_1 is the salt concentration of the buffer in the mixing vessel, C_2 is the salt concentration in the second vessel connected to the mixing vessel, V is the volume of the eluant and Vo is the volume in the mixing vessel which was maintained 250 ml throughout the experiment.

Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis was performed at pH 8.3 by the method recommended by Davis (1964) using 7.5%

acrylamide. Siliconized gel tubes (0.5 X 10 cm) were held vertically with their lower ends closed by rubber stoppers. Nearly 2.5 ml of small pore gel was poured into each tube. The tubes were allowed to stand for 30 minutes at room temperature to ensure polymerization. A suitable aliquot of enzyme sample (densed with 20% sucrose) was applied onto the surface of the gels. Electrophoresis was carried out for 2 hours or 5 hours using a current of 4 mA/tube, after which the gels were removed and stained with 1% (W/V) Amido Schwartz in 7% (V/V) acetic acid for 30 minutes. The gels were destained by repeatedly washing with 7% (V/V) acetic acid.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis:

Dodecylsulfate gel electrophoresis was carried out by the method of Laemmli (1970). The samples (1 mg/ml) were denatured at 60°C for 3 hours in a solution containing 3% sodium dodecylsulfate and 5% 2-mercaptoethanol. For electrophoresis 20 µl of this solution was mixed with few drops of glycerol and 5 µl of 0.1% bromophenol blue. Electrophoresis was carried out for 3 hours in 0.01M phosphate buffer, pH 7.0, containing 0.3% SDS by passing a current of 4 mA/tube. At the end of the run the dye front was marked by inserting a copper wire. The gels were stained with coomassie blue for 2 hours and destained in a mixture of 5% (V/V) methanol and 7.5% (V/V) acetic acid. After complete destaining, the distance of the protein migration and dye was measured. The relative mobility was determined according to the following equation:

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye front}} \quad \ldots (2)$$

CHAPTER III

R E S U L T S

R E S U L T S

Protease Activity During Larval Development:

The change in proteolytic activity at different larval ages after hatching from the eggs is shown in Fig. 1. Maximum activity was observed on the 9th day which declined subsequently till day 11. The pattern of changes in protein concentration in the gut was parallel to the protease activity. However, at 10 and 11 days, the decrease in proteolytic activity was slightly more in comparison to the decrease in protein concentration.

Protease Activity During Starvation:

The change in protease activity during starvation is shown in Fig. 2. There was an abrupt increase in enzyme activity at 4 hours of starvation, after which a continuous decrease in the activity was observed till 24 hours. The protein concentration of gut contents was found to decrease rapidly upto 4 hours of starvation followed by a slower decline till 24 hours. Thus, the specific activity was increased two-fold at 4 hours and subsequently declined on further starvation.

Effect of pH on Protease Activity of Crude Enzyme Solution:

The pH-activity curve is presented in Fig. 3. Since it was difficult to prepare casein solution below pH 6.0, or acid denatured

Figure - 1 : Protease activity during development of larvae of *Spodoptera litura*.

The protease activity and protein concentration of gut contents at different larval ages were determined as described in the text.

Protease activity ●

Protein concentration o

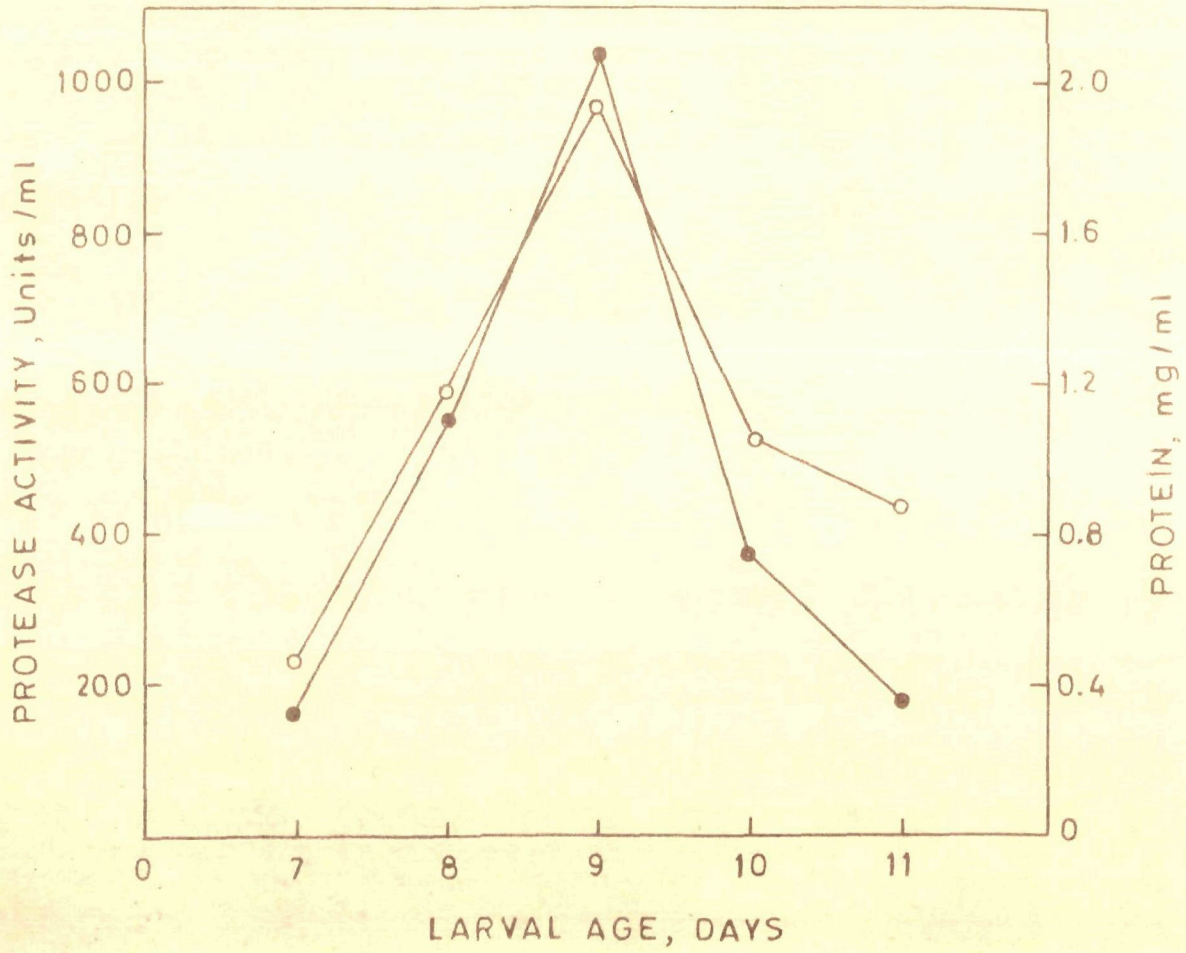

PROTEASE ACTIVITY, Units/ml
1000
800
600
400
200
0
PROTEIN, mg/ml
2.0
1.6
1.2
0.8
0.4
0
7
8
9
10
11
LARVAL AGE, DAYS

Figure - 2 : Protease activity during starvation period of larvae.

The fifth instar larvae were starved for varying time intervals; the protease activity and protein concentration of gut contents were determined. See text for details.

Protease activity ●

Protein concentration ○

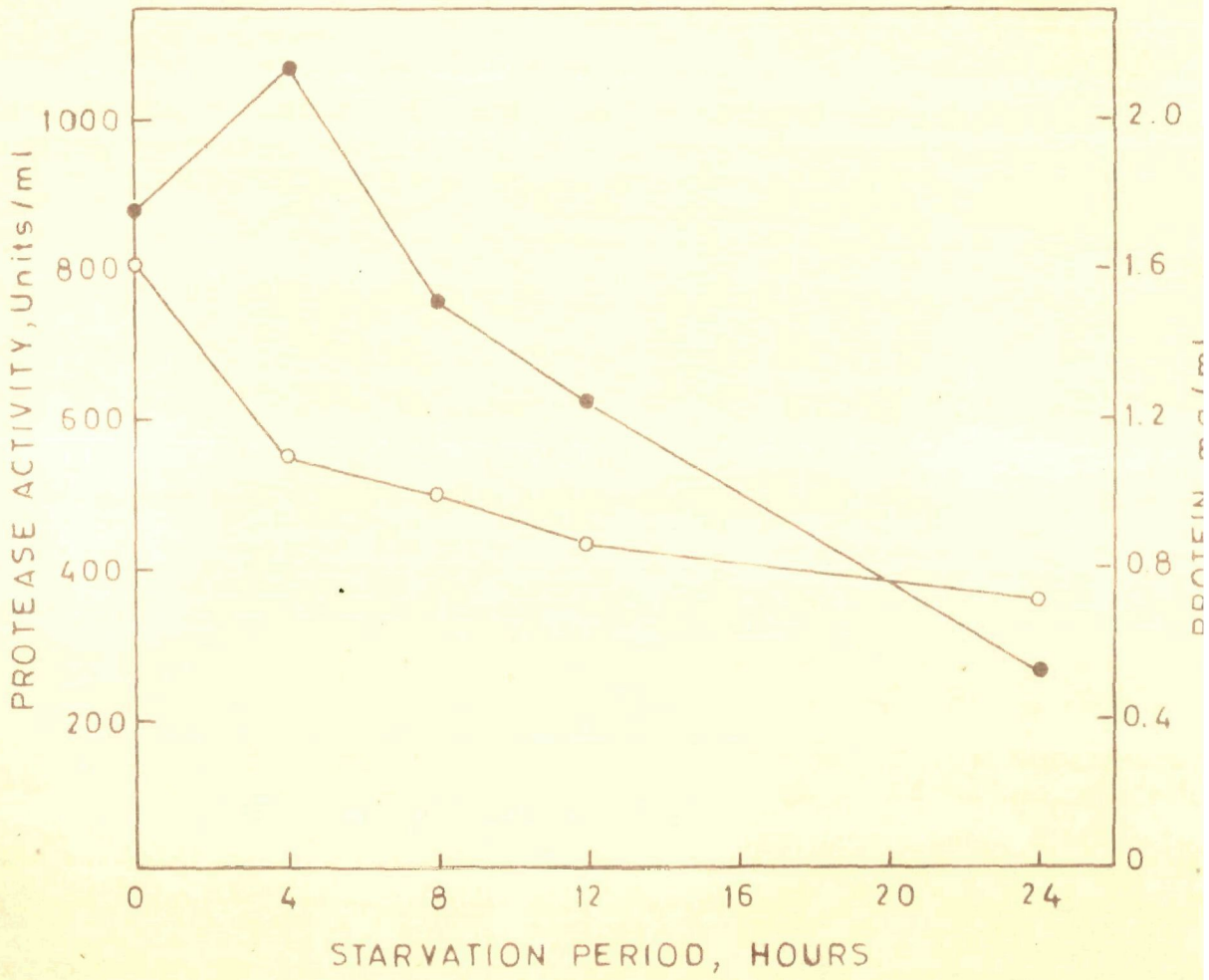
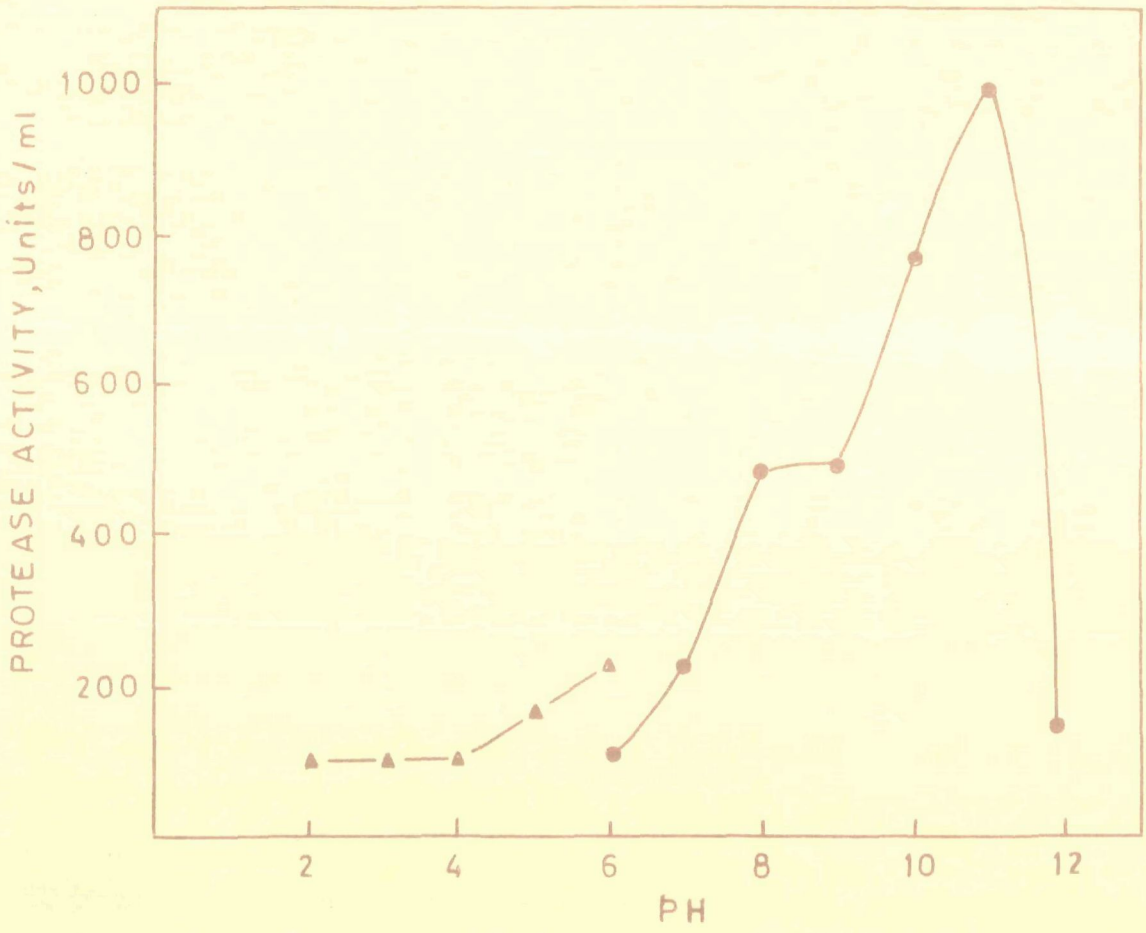

PROTEASE ACTIVITY, Units/ml
1000
800
600
400
200
2.0
1.6
1.2
0.8
0.4
0
0
4
8
12
16
20
24
STARVATION PERIOD, HOURS

Figure - 3 : Effect of pH on the protease activity of crude enzyme solution.

The reaction mixture in a total volume of 1 ml, contained 30 µg enzyme protein, 40 µmoles buffer and 10 mg casein dissolved in buffer of desired pH. After incubation for 15 minutes at 40°C, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The protease activity was determined as described in the text. Buffers used were: KH_2PO_4 - HCl (pH 2.0 - 3.0), K_2HPO_4 - HCl (pH 4.0 - 5.0), KH_2PO_4 - K_2HPO_4 (pH 6.0 to 8.0), KH_2PO_4 - NaOH (pH 9.0) and K_2HPO_4 - NaOH (pH 10.0 - 12.0).

Hydrolysis of acid denatured hemoglobin ▲

Hydrolysis of casein ●


PROTEASE ACTIVITY, Units/ml
1000
800
600
400
200
2
4
6
8
10
12
PH

haemoglobin solution above pH 6.0, haemoglobin was used as a substrate from pH 2.0 to 6.0 and casein was used between pH 6.0 to pH 12.0. No enzyme activity was observed below pH 4.0. The activity of the enzyme increased until pH 11.0, with a shoulder at pH 8.0, and declined sharply above pH 11.0.

Changes in Protease Activity During Incubation of Crude Enzyme Solution:

Crude enzyme solution was incubated at 37 ± 1°C for 55 hours and the specific activity was determined at different intervals (Table I). There was a sharp decrease in protein concentration until 22 hours of incubation without any significant change until 55 hours. The enzyme activity remained almost unaltered till 22 hours but subsequently a loss in activity was observed and by 55 hours, 37% of the activity was lost. Thus, incubation of the enzyme solution for 22 hours resulted in a three-fold increase in the specific activity which, however, declined on further incubation.

Effect of Dialysis on Protease Activity:

Crude enzyme solution was dialysed for 24 hours against 0.1M glycine-NaOH buffer, pH 11.0, at 4°C and 37°C. As shown in Table II, there was a 42% loss in enzyme activity when the enzyme solution was dialysed at 37°C, whereas only 10% loss in activity was observed at 4°C. In a separate experiment, it was observed that the enzyme activity was completely lost after 40 hours

TABLE - I

PROTEASE ACTIVITY DURING INCUBATION OF CRUDE ENZYME SOLUTION

Crude enzyme solution was incubated at 37°C, pH 11.0, and the aliquotes were taken at different intervals of time to measure the enzyme activity by standard method as described in the text. Protein was determined according to the method of Khanna et al. as described in the text.

Incubation period (hours)	Protein (mg/ml)	Activity (Units/ml)	Specific activity (Units/mg)
0	3.67	937	225
8	1.35	851	630
22	1.15	851	740
30	1.15	741	644
55	1.12	592	528

TABLE - II

EFFECT OF DIALYSIS ON PROTEASE ACTIVITY

Crude enzyme solution prepared in 0.1M glycin-NaOH buffer, pH 11.0, was dialysed at 37°C and 4°C for 24 hours. Suitable aliquots were taken to measure the enzyme activity by the standard method as described in the text.

Preparation	Activity (Units/ml)	% Inactivation
Undialysed	552	0
Dialysed at 4°C	497	10
Dialysed at 37°C	321	42

dialysis at 37°C. It is of interest to mention here that incubation of the enzyme solution for about 24 hours at 37°C caused very little inactivation.

Search for Zymogen:

Since many proteolytic enzymes from higher vertebrates (Boyer, 1971), lower vertebrates (Bradshaw et al., 1970; Reeck and Neurath, 1972), starfish (Camacho et al., 1970) and insects (Felsted et al., 1973; Ward, 1975) occur as inactive precursors, experiment was carried out to determine if any of the proteolytic enzymes in S. litura also occured as inactive zymogens. Crude extract in 0.1M Tris buffer, pH 8.0 was incubated at 40°C for 1,2,3 and 4 hours and the residual activity was assayed by the caseinolytic method. Since no increase in enzyme activity was observed, it was concluded that the enzyme does not exist as zymogen.

ISOLATION AND PURIFICATION OF S. litura PROTEASES:

S. litura larvae were cultured according to the method described earlier. The fifth instar larvae were dissected and their intestines collected in an ice cold beaker. Their contents were squashed out by means of a glass rod in 0.1M Tris buffer, pH 8.0. The mixture was centrifuged in cold at 4000 r.p.m. for 20 minutes and the supernatant was used as crude extract.

Initially the enzyme was partially purified to 7-fold with 80% recovery in two steps comprising of incubation at 37°C for

22 hours and Sephadex G-75 permeation chromatography (Fig. 4). The major active peak was, however, not homogeneous and gave three sharp and well separated bands on PAG electrophoresis. The details have already been published (Zafeer et al., 1976). The pH-activity profile of partially purified enzyme did not show a shoulder at pH 8.0 which was present in the crude extract. These observations suggested the presence of more than one proteases in the gut of S. litura which might be obtained due to proteolysis by incubation of crude extract at 37°C for 22 hours. Therefore, the purification was modified from the previously reported method (Zafeer et al., 1976). The yield and degree of purity at each step of modified method of purification are summarized in Table III.

(a) Acetone Fractionation:

Crude extract contains dark brown pigments and phenols. Though, these pigments were removed at each step, acetone fractionation was the major step. When the crude extract was passed through the column, the pigments were strongly bound with the gel and caused a retardation in the flow rate. Moreover, to remove these pigments from the gel, continuous washing for one week was required. Hence, to remove these pigments and phenols, the enzyme was precipitated by the addition of a three-fold chilled acetone prior to chromatography. It was left for one hour at 4°C as such to ensure complete precipitation. The acetone precipitated enzyme was collected by centrifugation

ıre - 4 : Chromatography on Sephadex G-75 column I.

The column (2.0 X 35 cm) was equilibrated with 0.05M Tris buffer, pH 8.0. About 38 mg protein, obtained by incubation of crude extract at 37°C, was applied and 3 ml fractions were collected at a flow rate of 20 ml/hr. See text for details.

Protease activity ●

Protein concentration ○

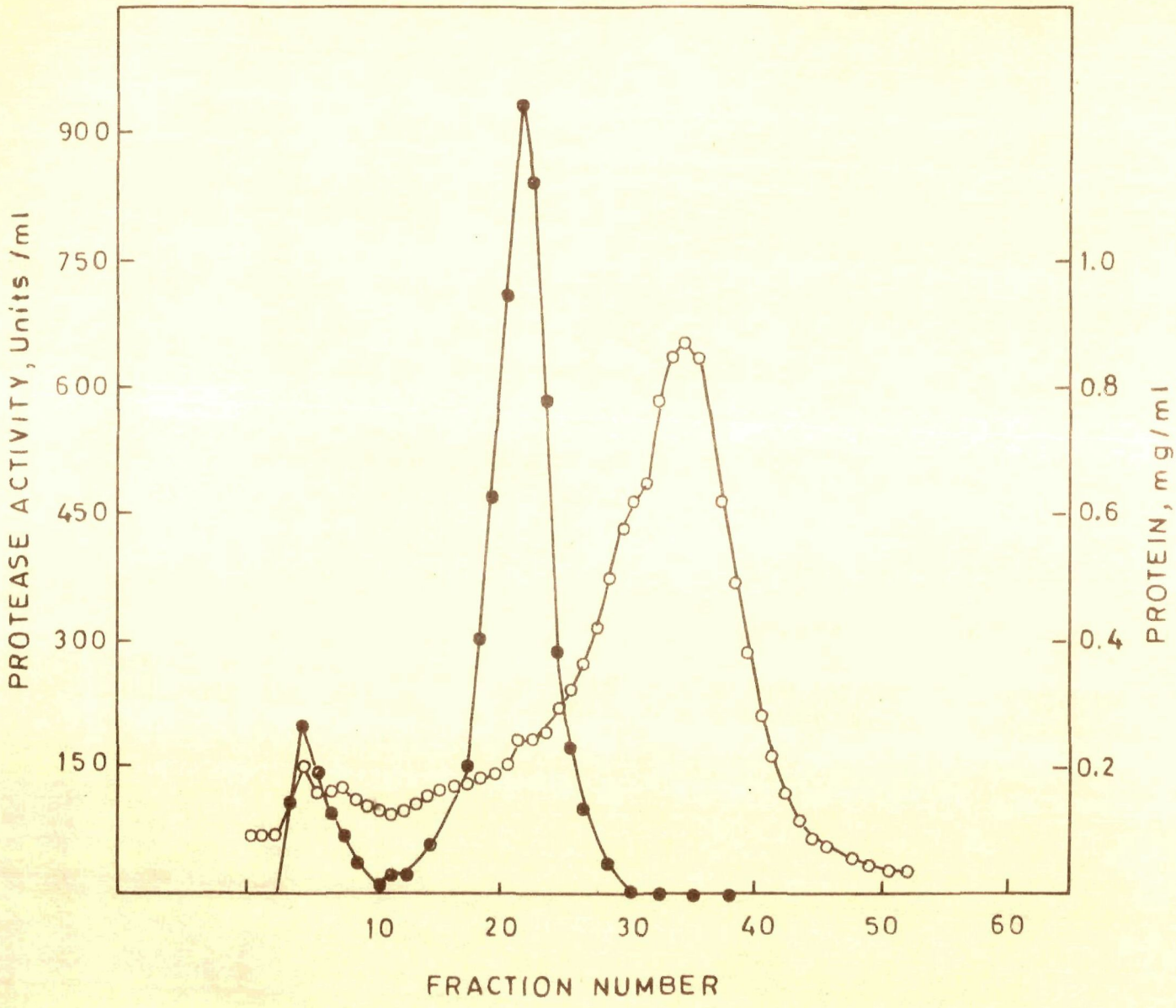

PROTEASE ACTIVITY, Units /ml
900
750
600
450
300
150
1.0
0.8
0.6
0.4
0.2
PROTEIN, mg/ml
10
20
30
40
50
60
FRACTION NUMBER

TABLE - III

PURIFICATION OF *S. litura* PROTEASES

Purification Step	Total Protein (mg)	Total Activity (Units X 10^{-2})	Specific Activity (Units X 10^{-2}/mg)	Yield (%)	Purification (Fold)
Crude extract	348	126.08	0.33	100.0	1
Acetone fractionation	294	93.14	0.31	74.0	1
Chromatography on Sephadex G-75	130	85.00	0.65	67.4	2
Chromatography on DEAE-Sephadex A-50					
P_I	8.2	23.65	2.95	18.7	9
P_{II}	14.8	25.97	1.75	20.5	5
P_{III}	9.0	18.17	2.02	14.4	6
Rechromatography on DEAE-Sephadex A-50					
P_{II}	10.5	24.52	2.33	19.4	7
P_{III}	6.6	17.25	2.61	13.6	8

at 4000 r.p.m. for 20 minutes. The precipitate was dissolved in 0.05M Tris buffer, pH 8.0.

(b) Sephadex G-75 Chromatography:

The enzyme solution obtained by acetone fractionation was now loaded on a G-75 column (2.16 X 56 cm), equilibrated and eluted with 0.05M Tris buffer, pH 8.0. 3 ml fractions were collected with a flow rate of 20 ml/hr. A suitable aliquot was taken for enzyme assay using casein as substrate. The chromatographic pattern for this gel filtration is shown in Fig. 5. Only two fold purification was achieved but a considerable amount of dark coloured impurities were removed at this step. Active fractions were pooled and dialysed against 0.02M Tris buffer, pH 8.0 at $4^{o}C$ for 24 hours changing the buffer several times.

(c) DEAE-Sephadex A-50 Chromatography:

The dialysed solution was applied to a DEAE-Sephadex A-50 column (2.6 X 42 cm), equilibrated with 0.02M Tris buffer, pH 8.0. The column was first washed with 400 ml of the same buffer. The flow rate was 50 ml/hr and 8 ml fractions were collected. The column was then subjected to a salt gradient elution from 0.02M to 0.85M NaCl in the same buffer. 4 ml fractions were collected at a flow rate of 50 ml/hr. A suitable aliquot was taken for caseinolytic assay. The chromatographic profile is represented in Fig. 6. One of the protease which was

Figure - 5 : Chromatography on Sephadex G-75 column II.

The column (2.16 X 56 cm) was equilibrated with 0.05M Tris buffer, pH 8.0. About 30 mg protein, obtained by acetone fractionation was applied and 4 ml fractions were collected at a flow rate of 20 ml/hr.

Protease activity ●

Protein concentration o

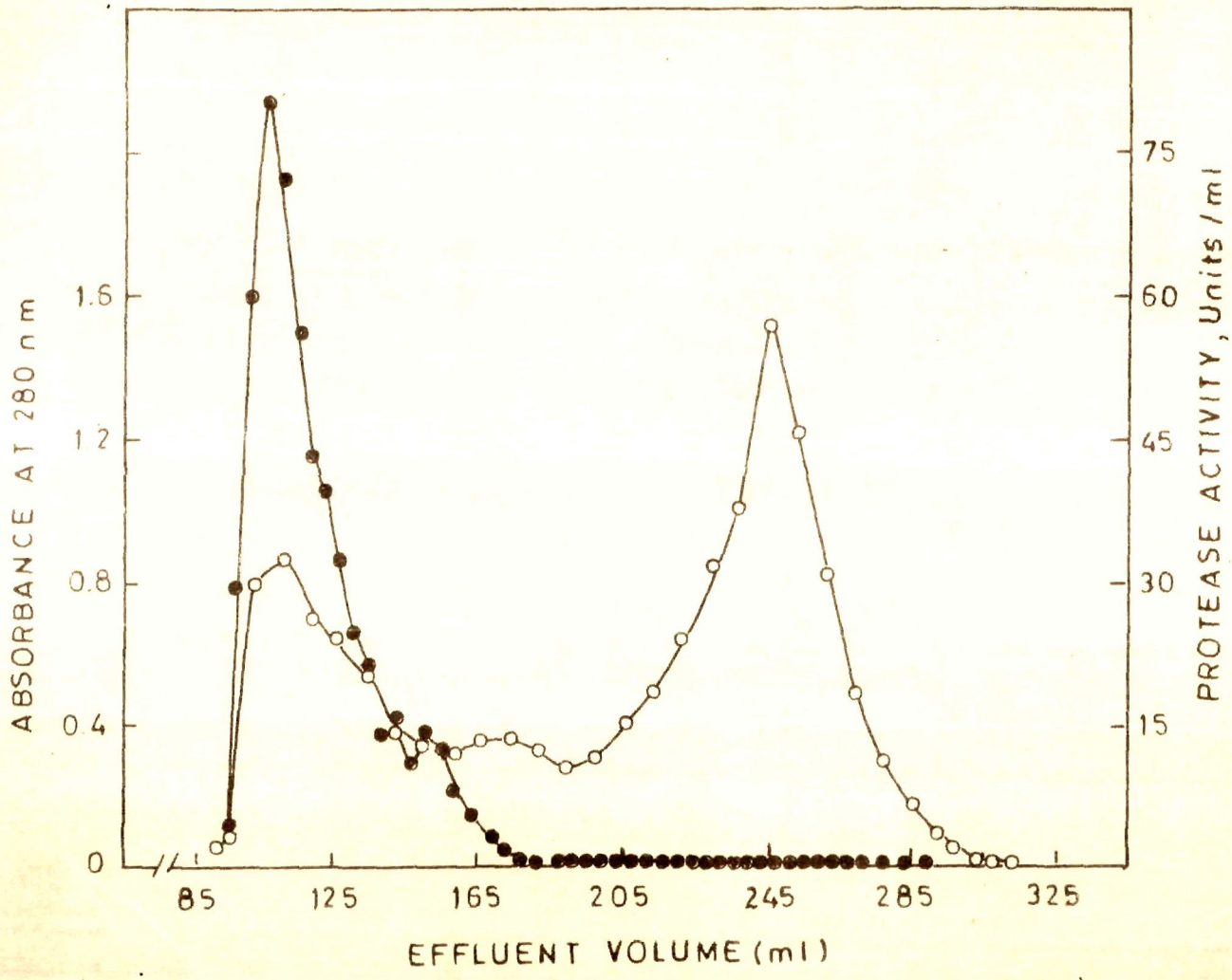
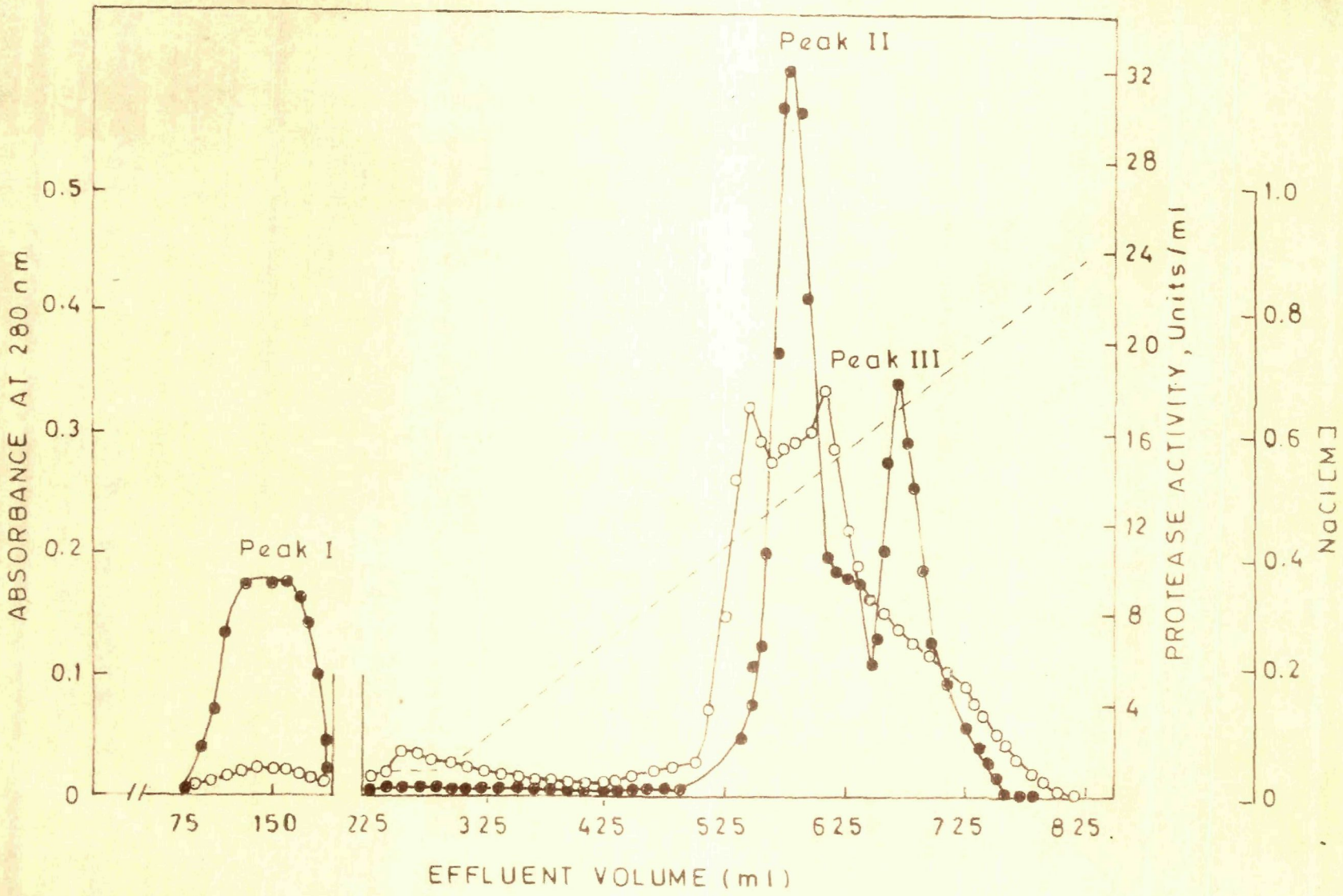

ABSORBANCE AT 280 nm
1.6
1.2
0.8
0.4
0
75
60
45
30
15
PROTEASE ACTIVITY, Units / ml
85
125
165
205
245
285
325
EFFLUENT VOLUME (ml)

Figure - 6 : DEAE-Sephadex Chromatography of *S. litura* alkaline proteases.

The column (2.6 X 42 cm) was equilibrated with about 2 litres of 0.02M Tris buffer, pH 8.0. About 130 mg protein in the same buffer was applied to the column. After washing the column with 200 ml 0.02M Tris buffer, pH 8.0, it was eluted with 0.02M - 0.085M Nacl and 4 ml fractions were collected at a flow rate of 50 ml/hr. ....., Nacl concentration in effluent.

Protease activity ●

Protein concentration o


Peak I
Peak II
Peak III
ABSORBANCE AT 280 nm
PROTEASE ACTIVITY, Units/ml
NaCl [M]
EFFLUENT VOLUME (ml)
0
0.1
0.2
0.3
0.4
0.5
4
8
12
16
20
24
28
32
0.2
0.4
0.6
0.8
1.0
75
150
225
325
425
525
625
725
825

not absorbed to the gel and eluted without retardation was designated as Protease I. Two other proteases which were resolved by salt gradient elution were known as Protease II and III. These two peaks were respectively eluted at 0.55 and 0.7M salt concentration. Active fractions consisting of protease II and III were separately pooled and dialysed for 24 hours against 0.02M Tris buffer, pH 8.0. These two proteases were rechromatographed on DEAE-Sephadex column by the same procedure as above. The chromatographic pattern is shown in Fig. 7 and Fig. 8. The purification procedure was repeated 5 times. It was reproducible with the exception that protease III was not detectable two times.

Homogeneity:

The purified preparations of alkaline proteases (P_I - P_{III}) gave single band on polyacrylamide gel electrophoresis. Fig. 9A represents the migration of protease I - III for 2 hours and Fig. 9B represents the migration of protease I and II for 5 hours.

DETERMINATION OF MOLECULAR PARAMETERS OF *S. litura* PROTEASES:

The molecular weight of *S. litura* proteases were calculated by gel filtration according to the procedure of Andrews (1964) using Sephadex G-200 (2.16 X 48 cm) column, equilibrated with 20 mM Tris buffer, pH 8.0, containing 0.1M NaCl. The column was calibrated with four marker proteins, cytochrome C, α-chymotrypsinogen A, ovalbumin and bovine serum albumin. 5 - 10 mg of

Figure - 7 : Rechromatography of Peak II on DEAE-Sephadex A-50 column.

Conditions were the same as in Figure 6.

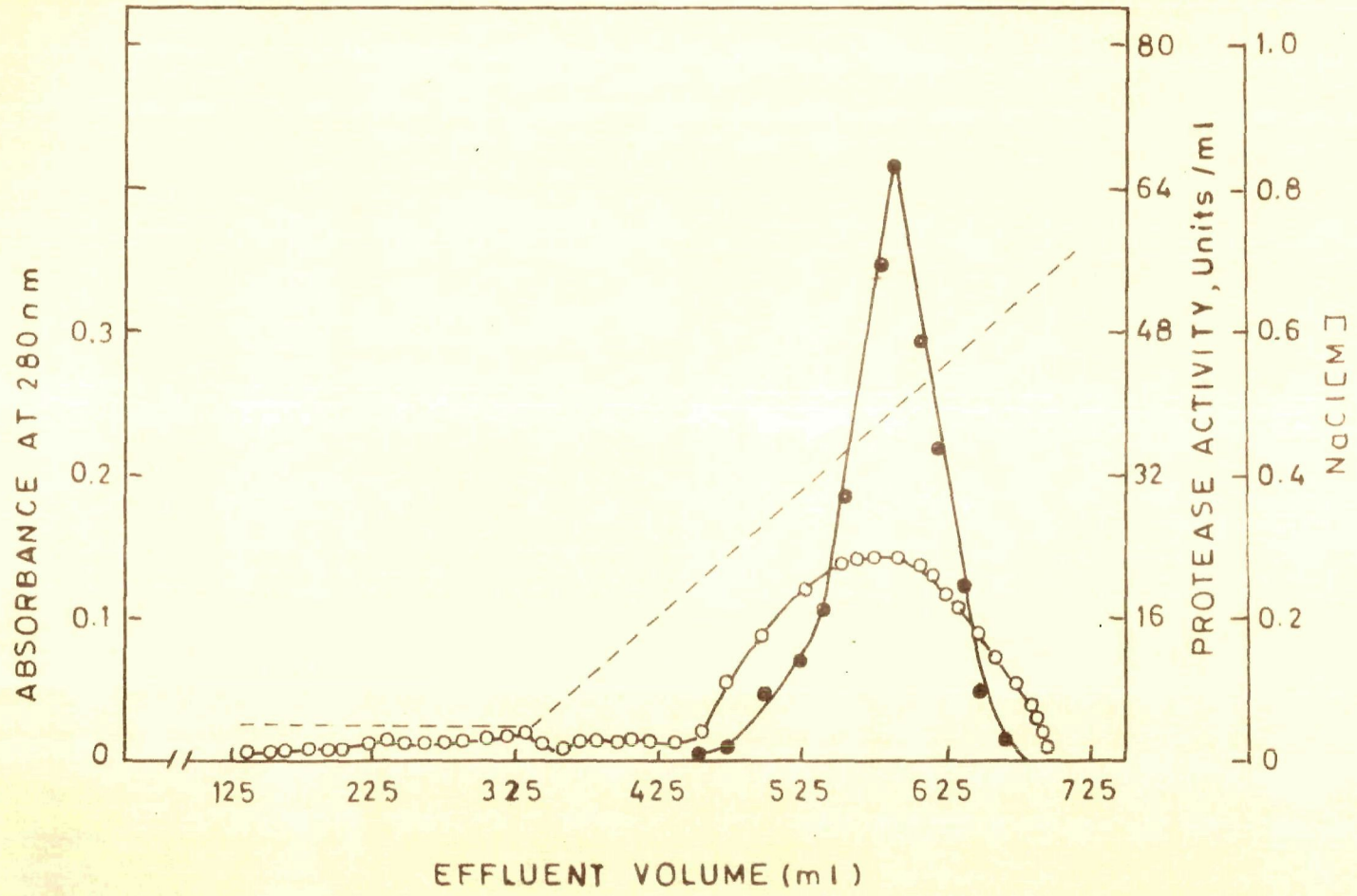

ABSORBANCE AT 280 nm
0
0.1
0.2
0.3
PROTEASE ACTIVITY, Units /ml
16
32
48
64
80
NaCl [M]
0
0.2
0.4
0.6
0.8
1.0
125
225
325
425
525
625
725
EFFLUENT VOLUME (ml)

Figure - 8 : Rechromatography of Peak III on DEAE-Sephadex A-50 column.

Conditions were the same as in Figure 6.

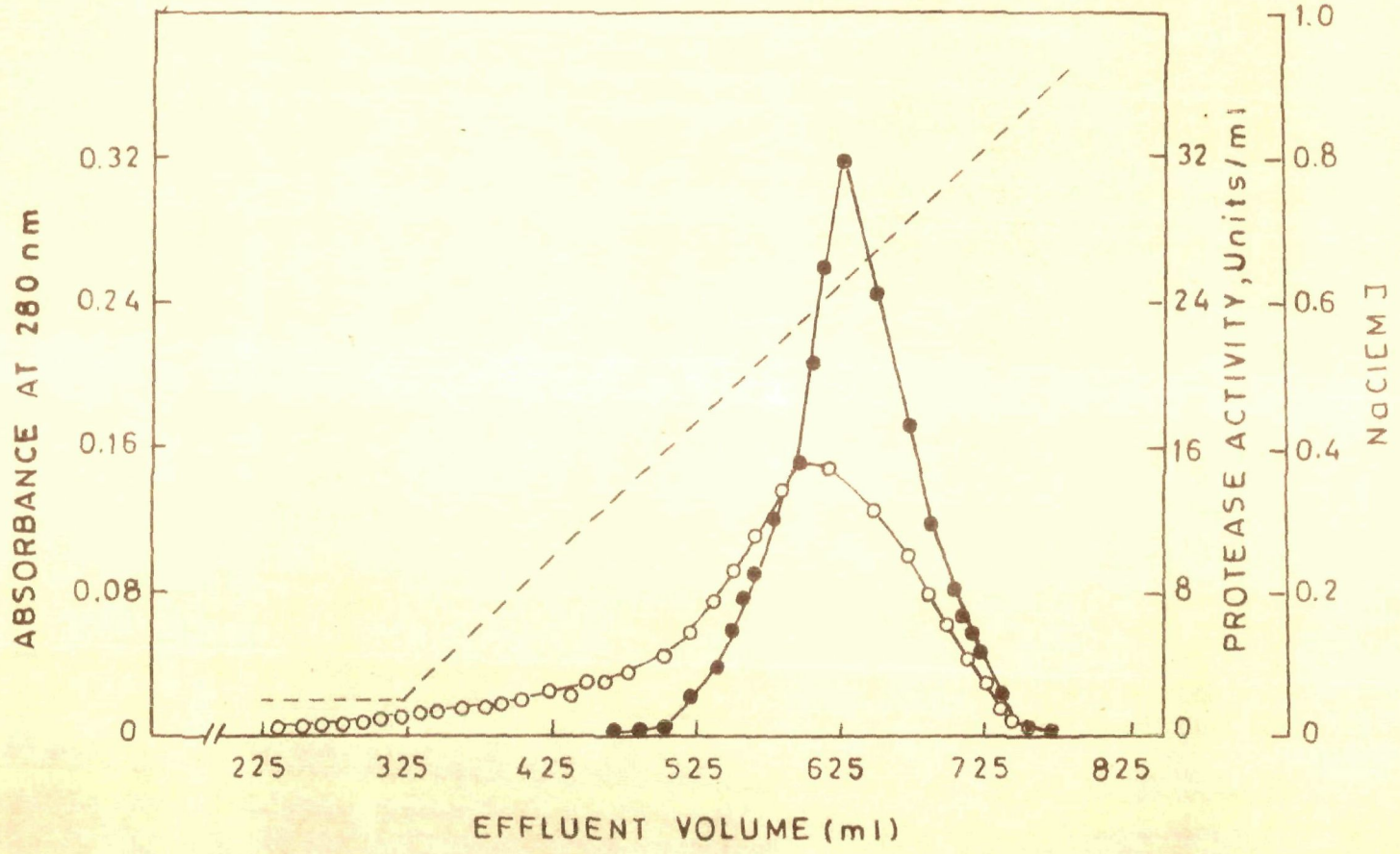

ABSORBANCE AT 280 nm
0.32
0.24
0.16
0.08
0
PROTEASE ACTIVITY, Units/ml
32
24
16
8
0
NaCl[M]
1.0
0.8
0.6
0.4
0.2
0
225
325
425
525
625
725
825
EFFLUENT VOLUME (ml)

Figure - 9A : Polyacrylamide gel electrophoresis of purified *S. litura* alkaline proteases.

7.5% gels with Tris-glycine buffer system, pH 8.3, were used. Protease I, A; protease II; B and protease III; C. Migration took place from the top (-) to bottom (+) for 2 hours. See text for details.

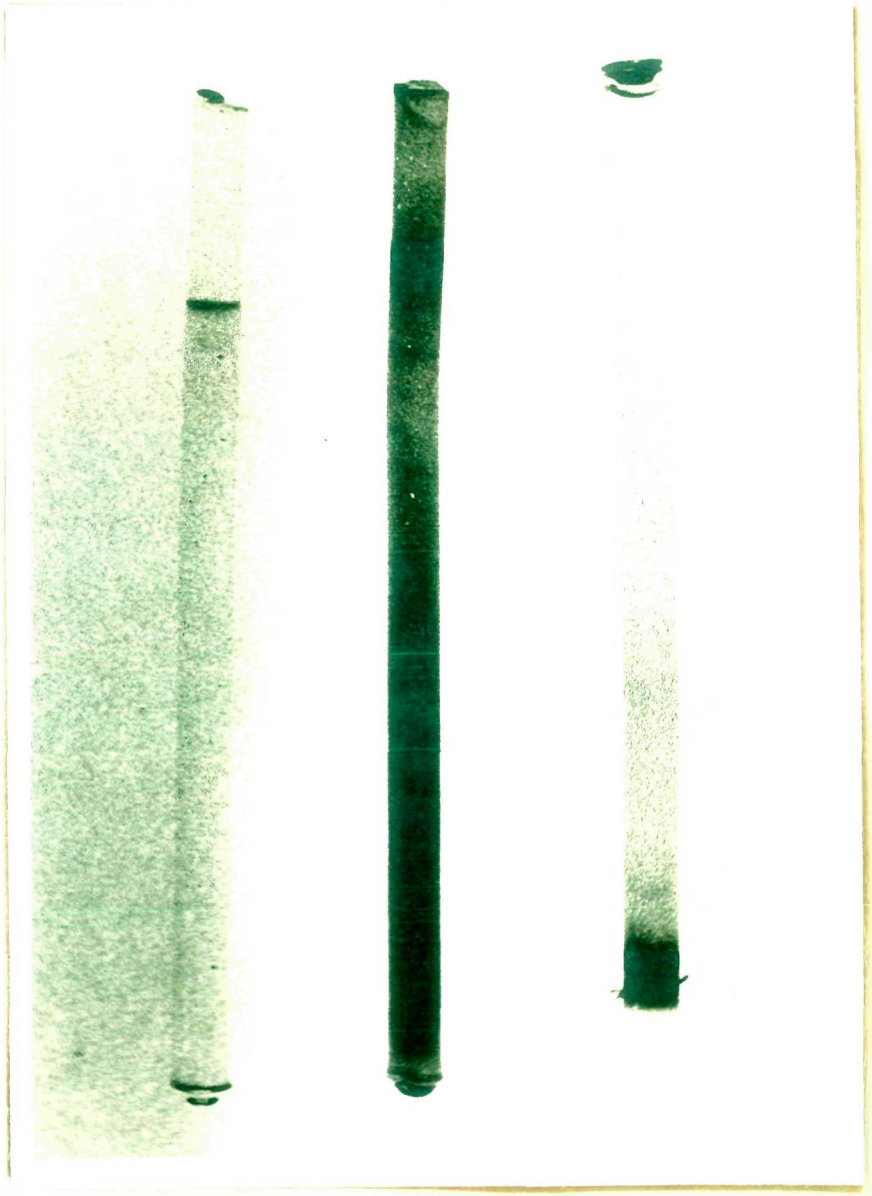
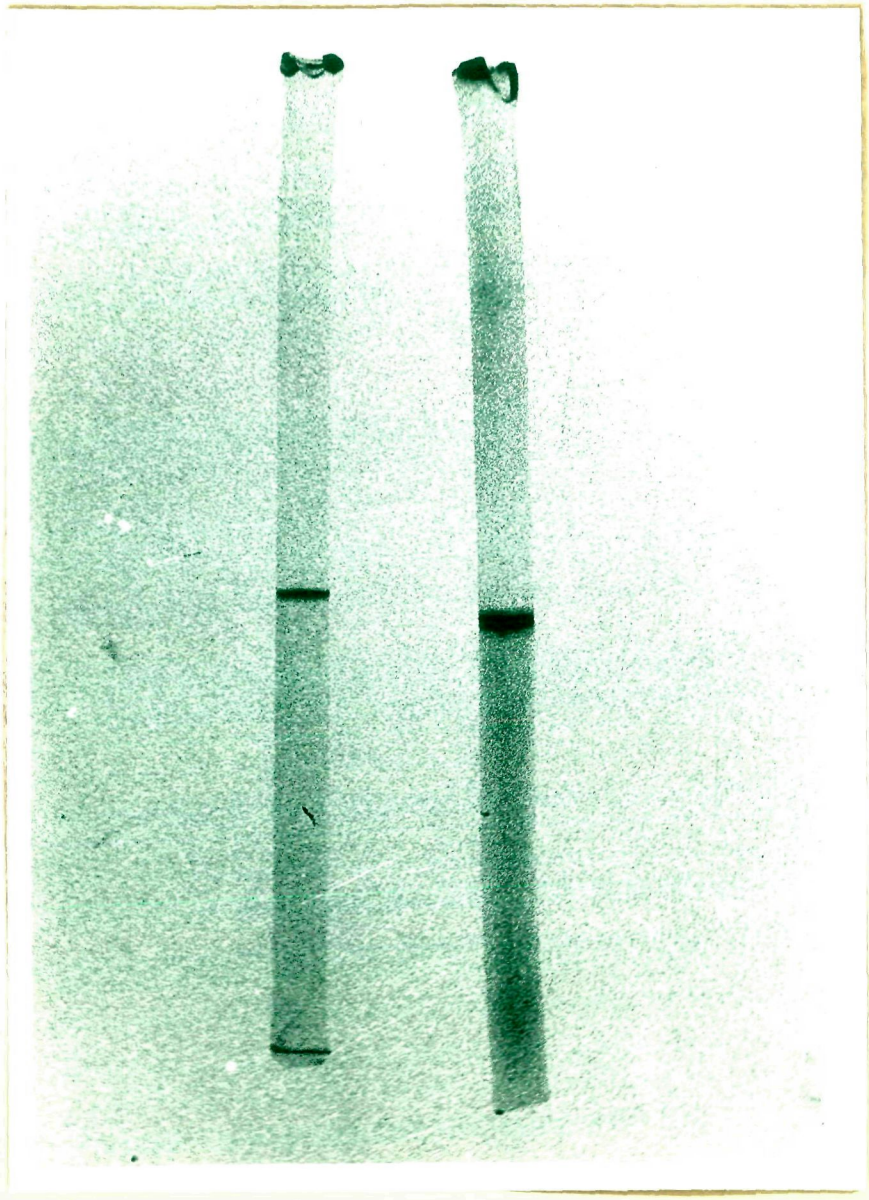

A
B
C

Figure - 9B : Polyacrylamide gel electrophoresis of protease I and II.

Conditions were same as in Figure 9A except that the migration time was for 5 hours. A represents protease I and B represents protease II.



A B

each protein was applied and eluted with the same buffer at a flow rate of 15 ml/hr and 2 ml fractions were collected. The proteins were determined by absorption either at 280 nm or at 660 nm using Lowry method. Blue dextran 2000 was measured at 625 nm. The enzyme activity for protease I - III was assayed by caseinolytic method. Elution profile for marker proteins and proteases is represented in Fig. 10 and 11 respectively. A plot of V_e/V_o versus logarithm of molecular weight (Fig. 12) according to the procedure of Andrew gave a straight line. Blue dextran was used to determine void volume (V_o) and tyrosine was used to determine inner volume (V_i). The total volume (V_t) was determined directly with water. The void volume was occasionally checked during column chromatography of marker proteins. No detectable change in V_o and V_t was found.

However, the elution position of a protein during gel filtration is better revealed to the Stokes' radius than to the molecular weight (Seigel and Monty, 1966; Ackers, 1964) and therefore, the Stokes' radius of S. litura proteases were determined. The data were processed using the following expressions:

$$K_d = \frac{Ve - Vo}{V_i} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

$$K_{av} = \frac{Ve - Vo}{V_t - V_o} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

where K_d is the distribution coefficient and K_{av} is the available distribution coefficient.

Figure - 10 : Gel filtration behaviour of standard proteins on Sephadex G-200 column.

About 5 - 10 mg of each standard proteins were chromatographed on the column (2.18 X 48 cm) which was equilibrated and eluted with 0.05M Tris buffer, pH 8.0 + 0.1M Nacl. 2 ml fractions were collected at a flow rate of 15 ml/hr.

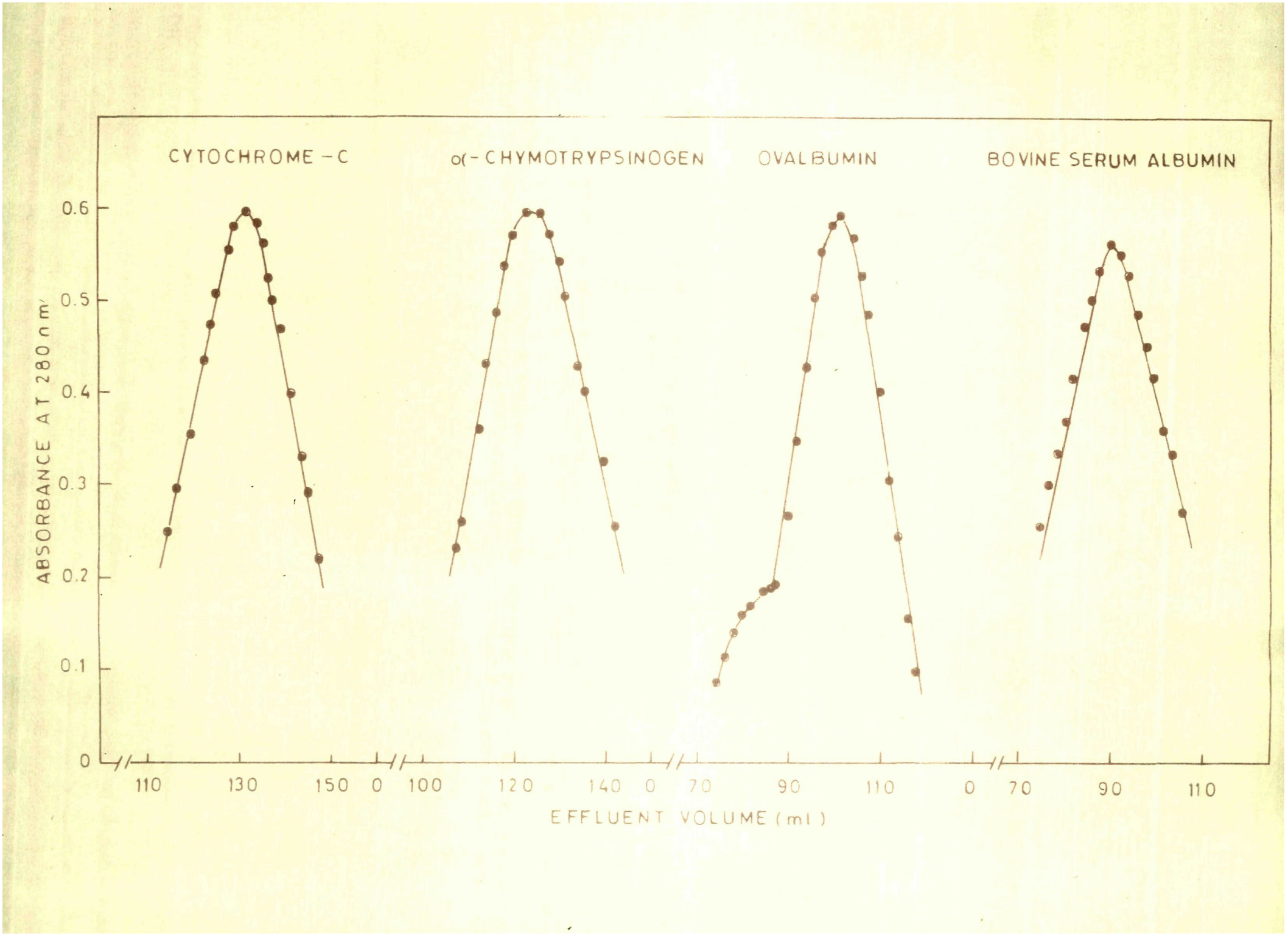

CYTOCHROME – C
α(– CHYMOTRYPSINOGEN
OVALBUMIN
BOVINE SERUM ALBUMIN
ABSORBANCE AT 280 n m
0.6
0.5
0.4
0.3
0.2
0.1
0
110
130
150
0
100
120
140
0
70
90
110
0
70
90
110
EFFLUENT VOLUME (ml)

Figure - 11 : Gel filtration behaviour of S. liture alkaline proteases on Sephadex G-200 column.

The experimental conditions were the same as described in Figure 10. Proteolytic activity of the eluate giving a clear maximum curve was checked by the standard method as described in the text.

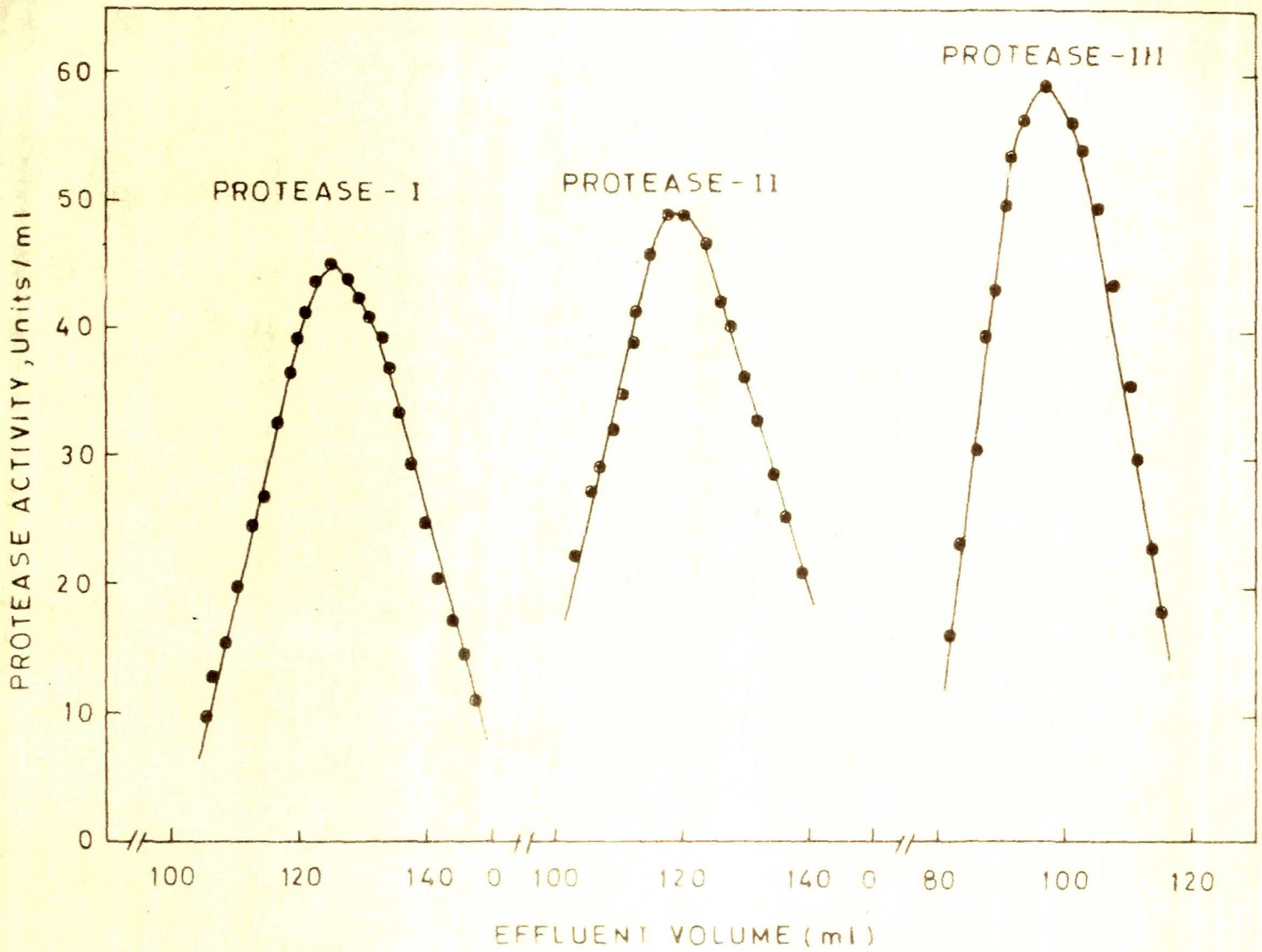
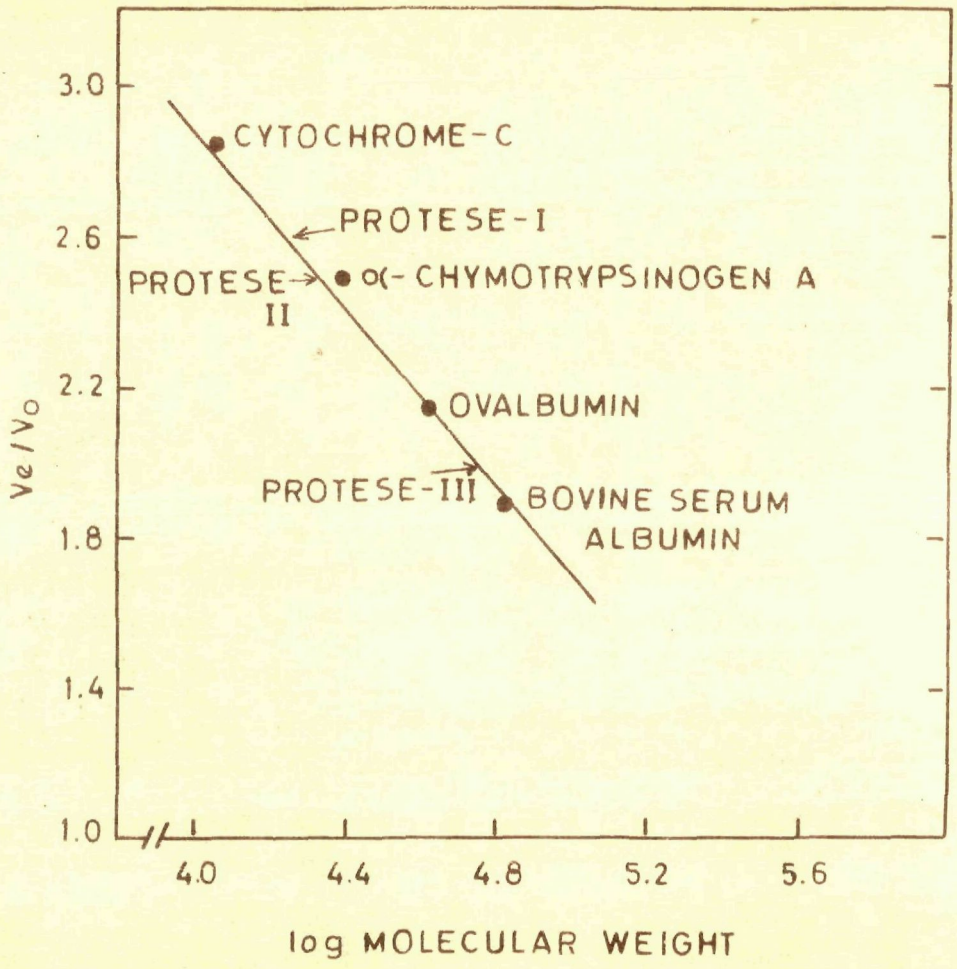

PROTEASE - I
PROTEASE - II
PROTEASE - III
PROTEASE ACTIVITY, Units / ml
0
10
20
30
40
50
60
100
120
140
0
100
120
140
0
80
100
120
EFFLUENT VOLUME (ml)

Figure 12 : Estimation of the molecular weight of S. litura proteases by gel filtration on Sephadex G-200.

Elution data of Table IV were treated according to the method of Andrews (1964). Plot of Ve/Vo Vs. log M gave straight line by the method of least squares.


3.0
2.6
2.2
1.8
1.4
1.0
Ve / Vo
CYTOCHROME-C
PROTESE-I
PROTESE II
α- CHYMOTRYPSINOGEN A
OVALBUMIN
PROTESE-III
BOVINE SERUM ALBUMIN
4.0
4.4
4.8
5.2
5.6
log MOLECULAR WEIGHT

The gel filtration data (Table IV) were analyzed using the procedures of Porath (1963), Laurent and Killander (1964) and Ackers (1964) by least squares method (Figs. 13-15).

The molecular weight values calculated by Andrews method were 16980 for protease I, 21380 for protease II and 53700 for protease III. Values of Stokes' radii obtained by correlation of Porath, Laurent and Killander and Ackers are summarized in Table V. The average values were 1.89 nm for protease I, 2.16 nm for protease II and 3.08 nm for protease III. The diffusion coefficients were calculated from the Stokes' radii using the Stokes-Einstein equation (Siegel and Monty, 1966):

$$D = \frac{KT}{6\pi\eta a}$$

where K is the Boltzmann constant (1.386×10^{-16} erg/degree), T is the absolute temperature ($303^{\circ}C$), and η is the coefficient of viscosity of the medium (0.0100 p for water and dilute aquous salt solutions at $20^{\circ}C$). Using the average values of Stoke's radii (Table V), diffusion coefficients for protease I - III were 11.79×10^{-7}, 10.32×10^{-7} and 7.24×10^{-7} cm^2/Sec.

Determination of Molecular Weight by SDS Polyacrylamide Gel Electrophoresis:

The molecular weight of S. Litura proteases were also determined by SDS gel electrophoresis at pH 7.0 according to the

TABLE - IV

GEL FILTRATION DATA OF *S. litura* ALKALINE PROTEASES AND STANDARD PROTEINS

Proteins	Ve/Vo	Stoke's radii (nm)	K_d	$(K_d)^{1/3}$	K_{av}	$(-\log K_{av})^{1/2}$	$erfc^{-1}K_d$
Cytochrome C	2.75	1.64	0.763	0.914	0.730	0.37	0.21
-Chymo-trypsinogen A	2.50	2.24	0.654	0.868	0.626	0.46	0.32
Ovalbumin	2.12	2.73	0.490	0.788	0.469	0.58	0.49
Bovine serum albumin.	1.89	3.55	0.390	0.708	0.373	0.65	0.61
Protease I	2.60	-	0.700	0.888	0.669	0.42	0.27
Protease II	2.48	-	0.645	0.864	0.608	0.47	0.33
Protease III	2.02	-	0.445	0.763	0.426	0.61	0.54

Figure - 13 : Estimation of the Stokes' radius of S. liture proteases.

The gel filtration data of Table IV were treated according to the correlation of Porath (1963). Plot of $(K_d)^{1/3}$ Vs. Stokes' radius gave straight line by the method of least squares.

Protease I o

Protease II ▲

Protease III Δ

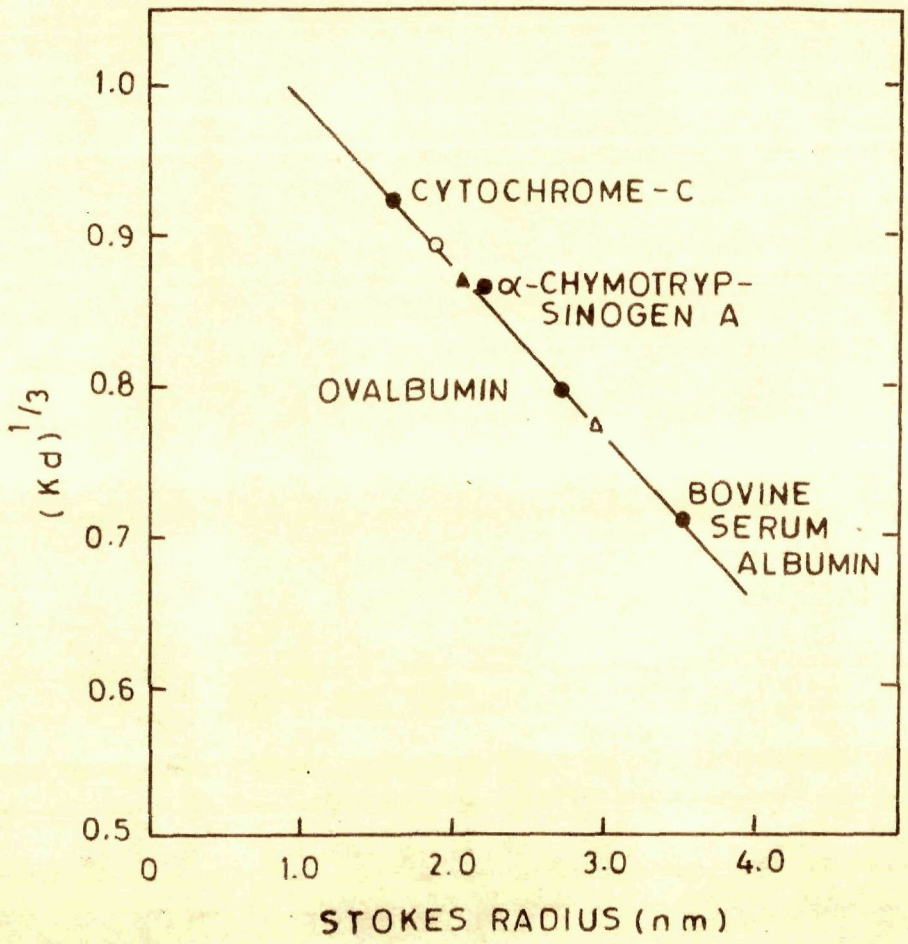

1.0
0.9
0.8
0.7
0.6
0.5
(Kd)^1/3
CYTOCHROME-C
α-CHYMOTRYP-
SINOGEN A
OVALBUMIN
BOVINE
SERUM
ALBUMIN
0
1.0
2.0
3.0
4.0
STOKES RADIUS (nm)

Figure - 14 : Estimation of the Stokes' radius of S. litura proteases.

The gel filtration data of Table IV were treated according to the correlation of Laurent and Killander (1964). Plot of $(-\log K_{av})^{\frac{1}{2}}$ Vs. Stokes' radius gave the straight line by the method of least squares.

Protease I ○

Protease II ▲

Protease III Δ

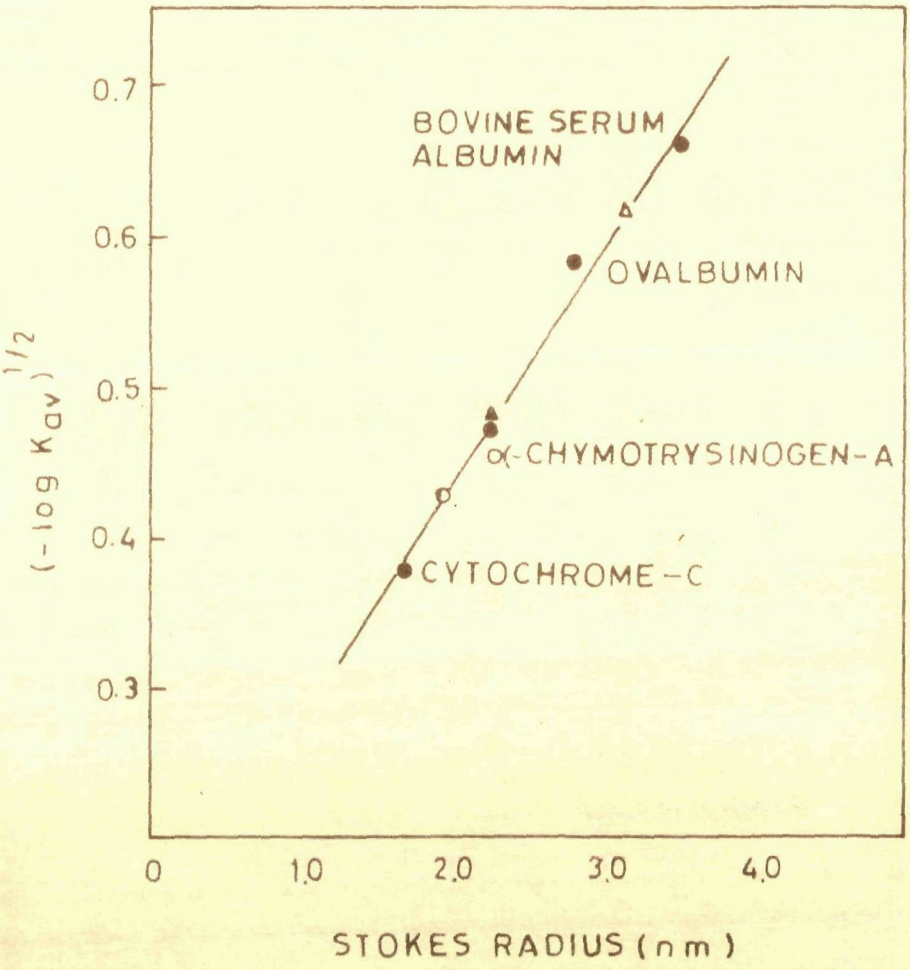

BOVINE SERUM ALBUMIN
OVALBUMIN
α-CHYMOTRYSINOGEN-A
CYTOCHROME-C
0.7
0.6
0.5
0.4
0.3
0
1.0
2.0
3.0
4.0
(-log Kav)^1/2
STOKES RADIUS (nm)

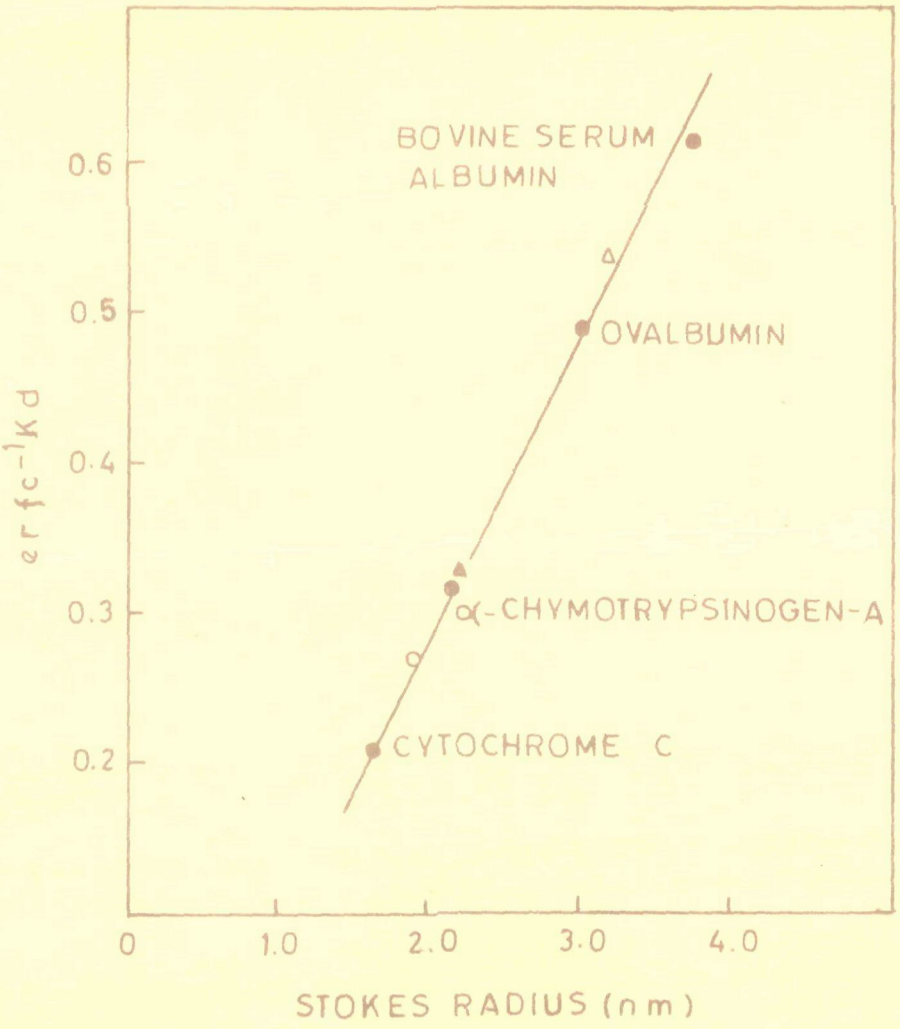
Figure - 15 : Estimation of Stokes' radius of *S. litura* proteases.

The gel filtration data of Table IV were plotted according to the correlation of Ackers (1967). The straight line was drawn by the method of least squares.

Protease I ○

Protease II ▲

Protease III Δ


BOVINE SERUM ALBUMIN
OVALBUMIN
α-CHYMOTRYPSINOGEN-A
CYTOCHROME C
0.6
0.5
0.4
0.3
0.2
erfc⁻¹Kd
0
1.0
2.0
3.0
4.0
STOKES RADIUS (nm)

method of Weber and Osborn (1969). The same marker proteins were used as in the caliberation of Sephadex G-200 column described above. The relative mobilities of each marker proteins were determined and graphed against the subunit molecular weight (Fig. 16). A least square analysis of the data indicated a linear relation between log M and relative mobility (Rm).

$$\log M = -1.94\ Rm + 6.22 \quad \ldots\ldots\ldots \quad (5)$$

S. litura proteases gave single band in SDS polyacrylamide gel electrophoresis with the Rm values of 0.975, 0.96 and 0.785 which correspond the molecular weights of 18500, 22910 and 50000 respectively. The physical parameters of S. litura proteases are summarized in Table V.

ENZYMATIC PROPERTIES OF S. litura PROTEASES:

Effect of Incubation Time:

Each proteases were incubated for different intervals of time at 40°C and pH 9.0. The enzyme activity was assayed by the caseinolytic method. As shown in Fig. 17, S. litura proteases showed a linear relationship between the enzyme activity and the incubation period upto 60 minutes.

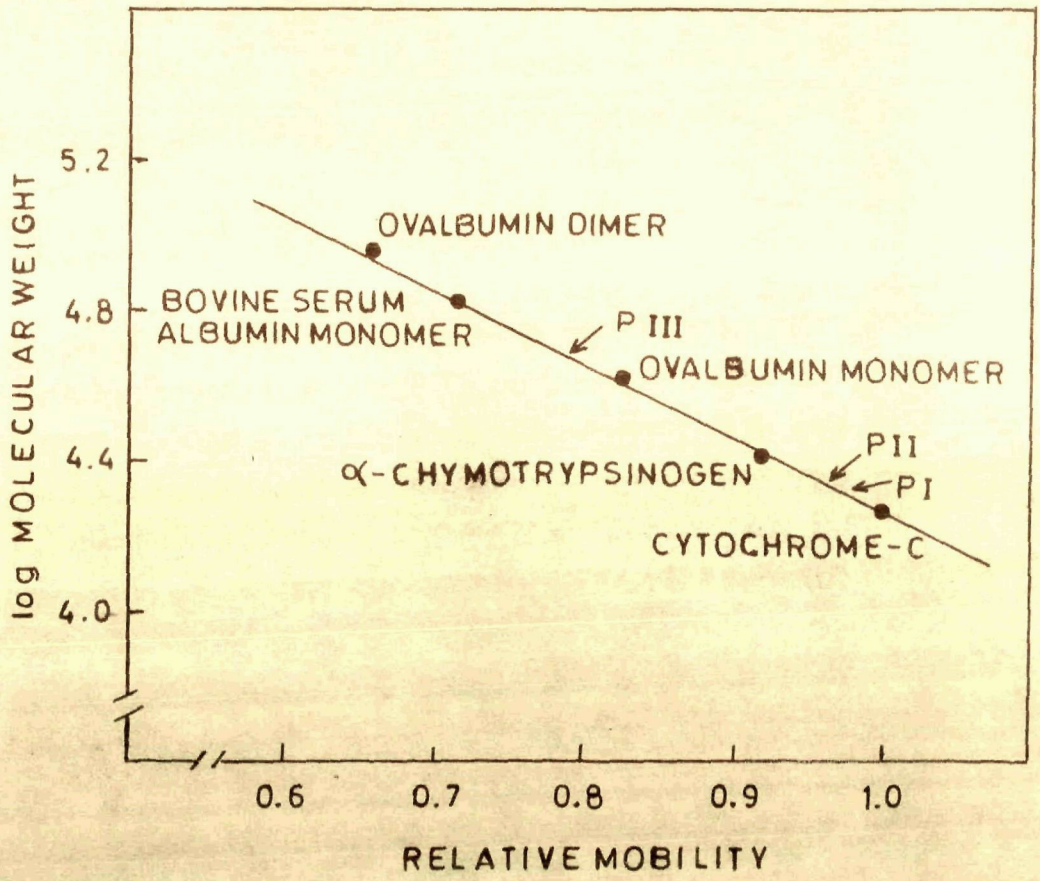

log MOLECULAR WEIGHT
5.2
4.8
4.4
4.0
OVALBUMIN DIMER
BOVINE SERUM ALBUMIN MONOMER
P III
OVALBUMIN MONOMER
P II
P I
α-CHYMOTRYPSINOGEN
CYTOCHROME-C
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY

Figure - 16 : Molecular weight determination of *S. litura* alkaline proteases by SDS gel electrophoresis.

Plot of the relative mobilities (Rm) of the standard proteins Vs. logarithm of their subunit molecular weight gave straight line by the method of least squares. See text for details.

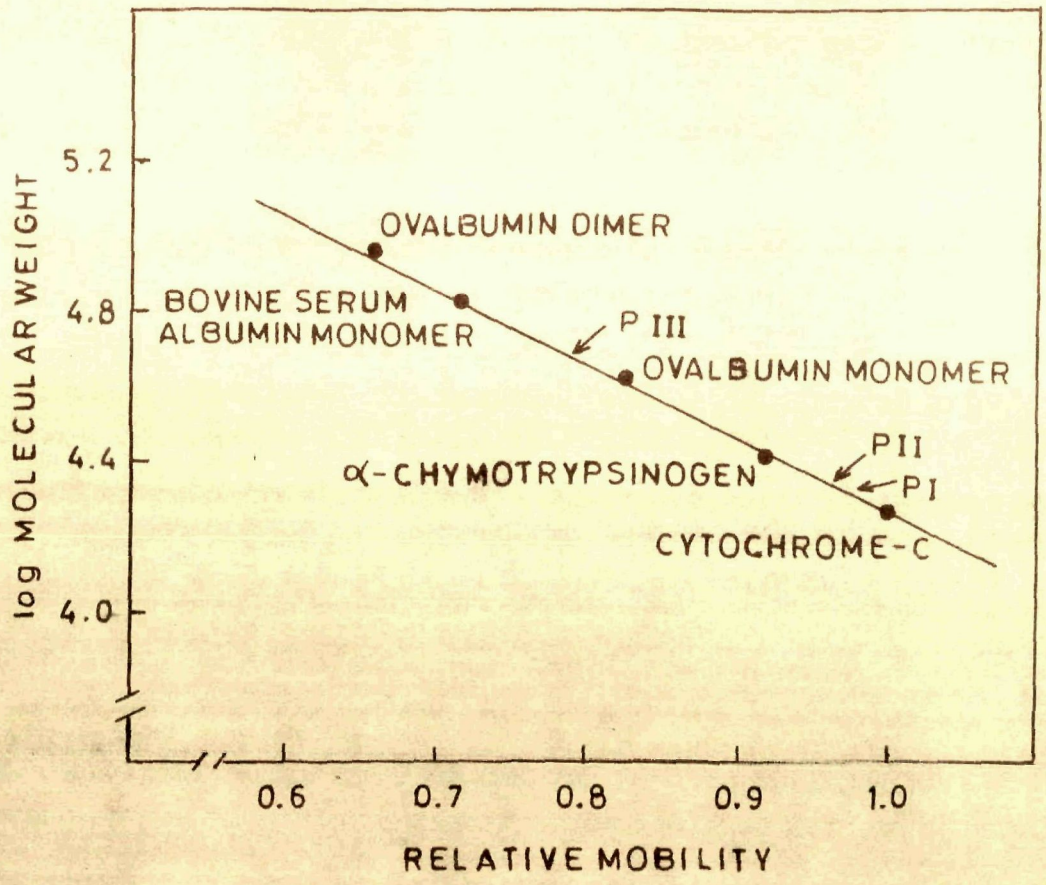

log MOLECULAR WEIGHT
5.2
4.8
4.4
4.0
OVALBUMIN DIMER
BOVINE SERUM ALBUMIN MONOMER
P III
OVALBUMIN MONOMER
PII
PI
α-CHYMOTRYPSINOGEN
CYTOCHROME-C
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY

TABLE - V

SUMMARY OF PHYSICO-CHEMICAL PROPERTIES OF *S. litura* PROTEASES

Parameter	Determination	VALUES		
		Protease I	Protease II	Protease III
Molecular weight	From gel filtration by Andrews method on Sephadex G-200.	16,980	21,380	53,700
	From SDS gel electrophoresis.	18,500	22,910	50,000
Stokes' radius (nm)	According to Porath.	1.85	2.10	2.90
	According to Laurent and Killander.	1.92	2.21	3.18
	According to Ackers.	1.91	2.18	3.15
	Average.	1.89	2.16	3.08
Diffusion coefficient, D (cm^2/Sec.)	From average Stokes' radius.	11.79×10^{-7}	10.32×10^{-7}	7.24×10^{-7}

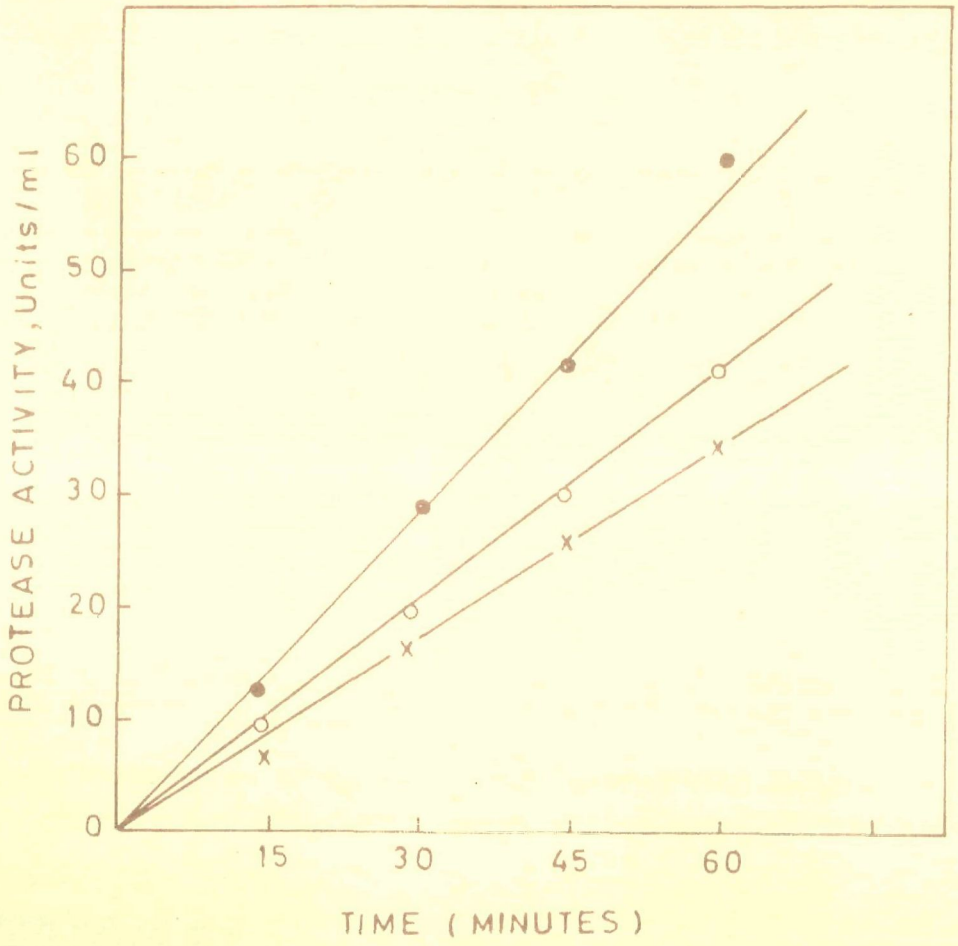
Figure - 17 : Effect of incubation time on enzyme activity of *S. litura* proteases.

Protease I - III were incubated at 40°C for different time intervals and the proteolytic activity was determined as described in the text.

Protease I ●

Protease II o

Protease III X


PROTEASE ACTIVITY, Units/ml
0
10
20
30
40
50
60
15
30
45
60
TIME (MINUTES)

pH Optima:

The pH-activity curves of the alkaline proteases for hydrolysis of casein are presented in Fig. 18. The pH optima for protease I - III were 11.0, 10.5 and 9.0 respectively.

Temperature Optima:

The results presented in Fig. 19 show that the optimum temperatures for protease I - III obtained by 20 minutes reaction at pH 8.0 were 60°C, 55°C and 50°C respectively. The kinetic parameters are summarized in Table VI.

pH-Stability:

The effect of pH on the stability of proteases is shown in Fig. 20. While protease I and II were relatively more stable at pH 2.4 retaining more than 60% of the initial activity after 45 minutes at this pH, the protease III had only 10% activity. The protease I and II retained full activity at pH 6.0 but the protease III shows some denaturation even at this pH. At pH 10.0, protease I - III had about 90% of initial activity. There was no remarkable decrease in activity of protease I and II at pH 12 but only 50% activity remained in protease III.

Figure - 18 : pH-activity profile for *S. litura* proteases.

The reaction mixture in a total volume of 1 ml contained 5-8 µg enzyme protein, 40 µmoles buffer and 10 mg casein dissolved in buffer of desired pH. After incubation for 20 minutes at 40°C, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The proteolytic activity was determined as described in the text. Buffers used were: phosphate (pH 6.0 to 8.0) and glycine-NaOH (pH 9.0 to 12.0).

Protease I ○

Protease II ▲

Protease III ×

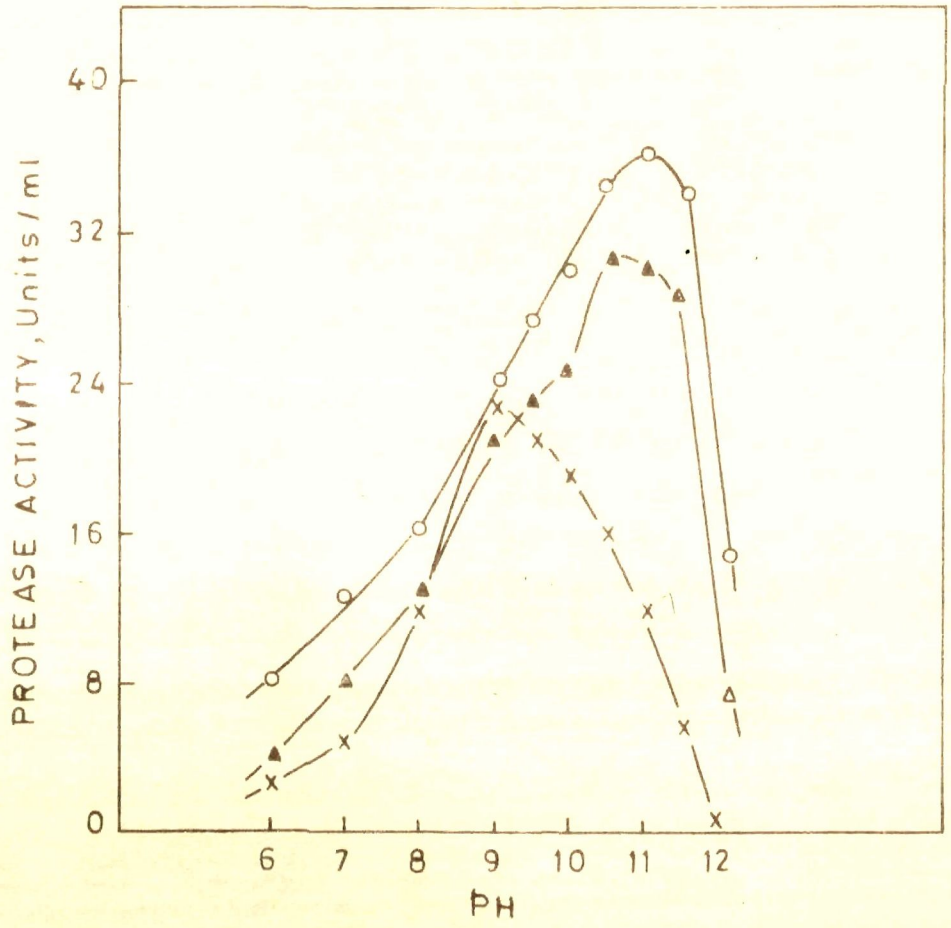

PROTEASE ACTIVITY, Units / ml
40
32
24
16
8
0
6
7
8
9
10
11
12
PH

Figure - 19 : Effect of temperature on the proteolytic activity of *S. litura* alkaline proteases.

The reaction mixture in a total volume of 1 ml contained 5-8 µg enzyme protein, 40 µmoles Tris buffer, pH 8.0 and 10 mg casein dissolved in the same buffer. After incubation at the indicated temperatures for 20 minutes, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The proteolytic activity was determined as described in the text.

Protease I ○

Protease II ▲

Protease III ×

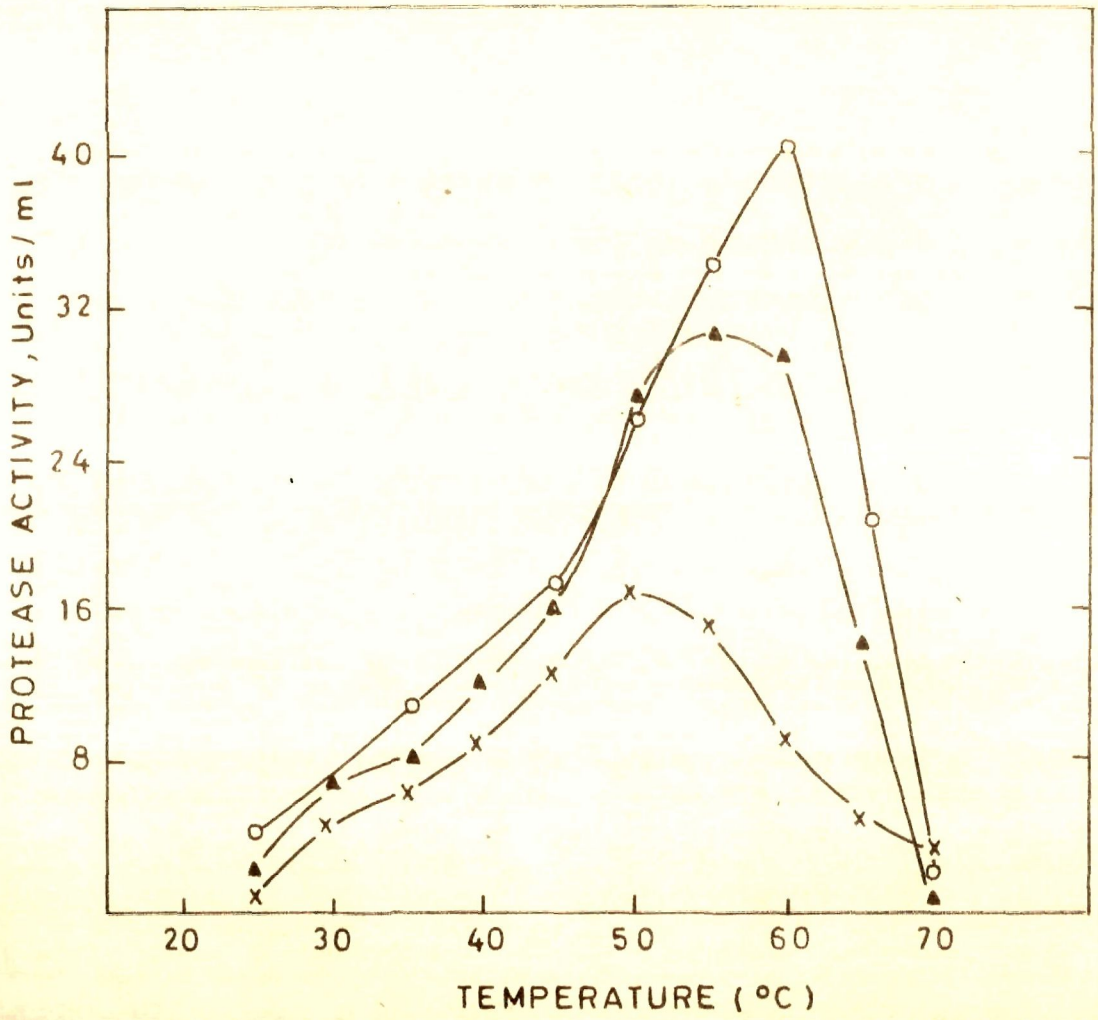

PROTEASE ACTIVITY, Units/ml
40
32
24
16
8
20
30
40
50
60
70
TEMPERATURE (°C)

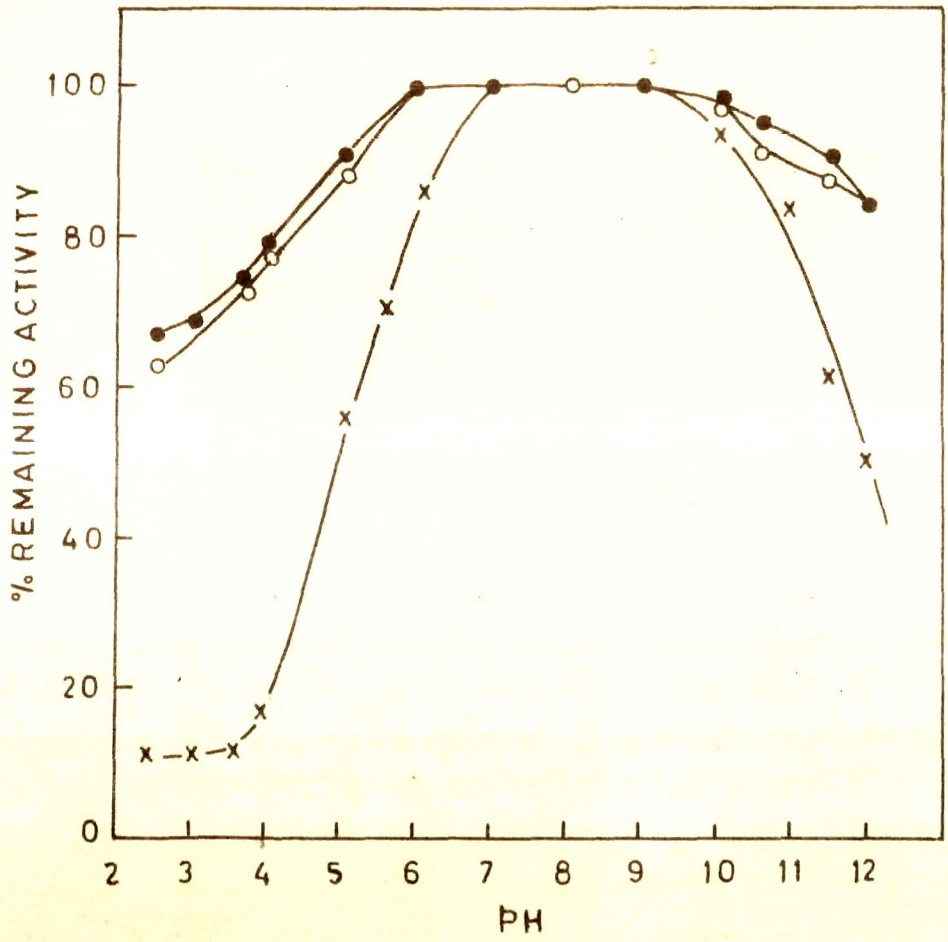
Figure - 20 : Effect of pH on stability of *S. litura* alkaline proteases.

5-8 µg enzyme protein and 40 µmoles buffer in a total volume of 0.5 ml was incubated at the indicated pH values for 45 minutes at 35°C. The pH of reaction mixtures were adjusted to pH 9.0 with 0.1M glycine-NaOH buffer and a suitable aliquot was taken to determine the caseinolytic activity by standard procedure. The activity at pH 9.0 was taken as 100 per cent. Buffers used were: glycine-HCl (pH 2.0 to 3.0), acetate (pH 3.5 to 5.0), phosphate (pH 5.5 to 8.0) and glycine-NaOH (pH 8.5 to 12.5).

Protease I ●

Protease II o

Protease III x


% REMAINING ACTIVITY
100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
11
12
PH

Temperature Stability:

Thermal stability measurements for each proteases is given in Fig. 21. There was no remarkable difference in terms of inactivation of all the three proteases. The inactivation began at 45°C, and at 60°C, almost all the proteases lost full activity. At 50°C, the protease I - III respectively had 94, 85 and 80% activity.

Lineweaver-Burk Plot:

Linear double reciprocal plots were obtained with each of the protease using casein as the substrate (Fig. 22). The plots were obtained by the least square method and the values of Km were computed from the intercepts and slopes of the linear plots and listed in Table VI. These values are 5.7×10^{-6} M for protease I, 2.9×10^{-6} M for protease II and 2.1×10^{-6} M for protease III.

Effect of Guanidine Hydrochloride:

Protease I - III were incubated with various concentrations of guanidine hydrochloride for 30 minutes at 35°C. Bovine pancreatic trypsin was also incubated under similar conditions for comparison. The residual peptidase activity was determined using BAPA as the substrate. Under the defined conditions, the denaturation effect of guanidine hydrochloride on proteases was

Figure - 21 : Thermal inactivation of S. litura alkaline proteases.

5-8 µg enzyme protein dissolved in 40 umoles Tris buffer, pH 8.0, was preincubated at the indicated temperatures for 20 minutes, immediately cooled to 0°C and the residual activity was assayed by the standard assay procedure. The activity at 40°C was taken as 100 per cent.

Protease I ●

Protease II O

Protease III x

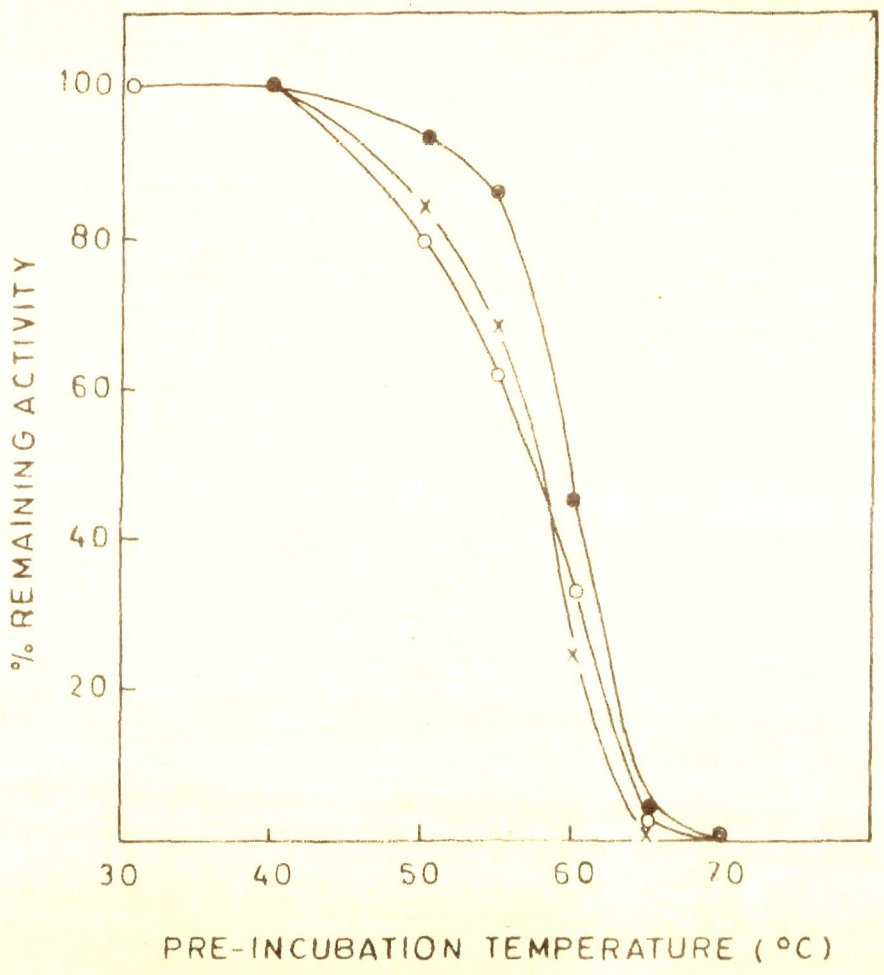

100
80
60
40
20
% REMAINING ACTIVITY
30
40
50
60
70
PRE-INCUBATION TEMPERATURE (°C)

Figure - 22 : Lineweaver-Burk plots of *S. litura* alkaline proteases with casein as a substrate.

The assay mixture in a total volume of 1 ml contained 5-8 µg enzyme protein. The substrate concentration was varied in the range 0.012×10^{-4} to 0.3×10^{-4} M, pH 9.0, 40°C. The enzyme activity was determined by the standard method as described in the text.

Protease I ○

Protease II ▲

Protease III ×

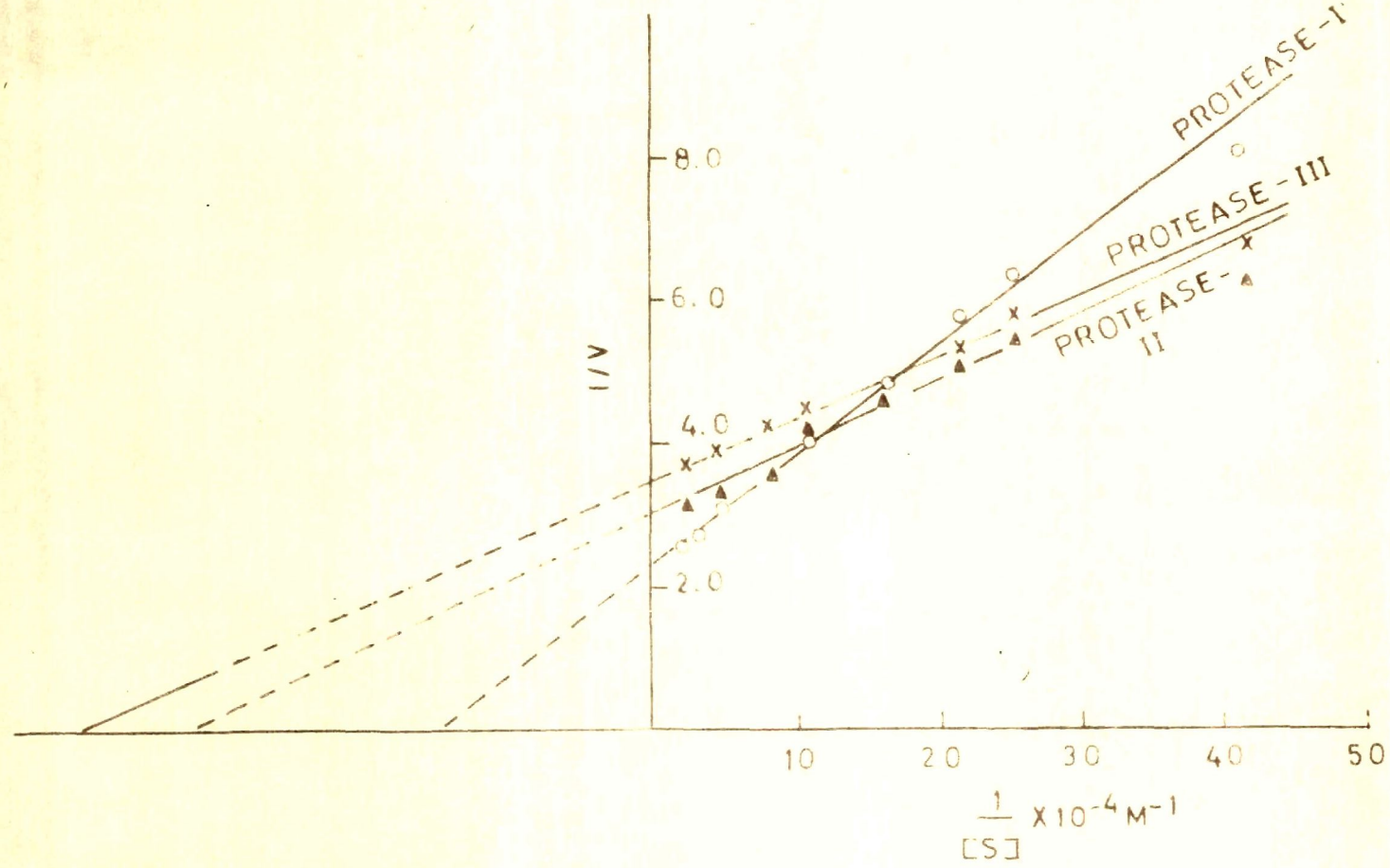

PROTEASE-I
PROTEASE-III
PROTEASE-II
8.0
6.0
4.0
2.0
1/v
10
20
30
40
50
1/[S] X10⁻⁴M⁻¹

TABLE - VI

KINETIC PROPERTIES OF *S. litura* PROTEASES

Enzyme	pH Optima	Temperature Optima (^{o}C)	Km (M^{-1})
Protease I	11.0	60	5.7×10^{-6}
Protease II	10.5	55	2.9×10^{-6}
Protease III	9.0	50	2.1×10^{-6}

almost similar to trypsin except in case of protease III which was relatively less susceptible to guanidine hydrochloride (Fig.23).

Effect of Metal Ions:

Effect of various metal ions on the S. litura proteases are summarized in Table VII. Even at relatively higher concentrations, no significant effect was observed with Mg^{++}, K^{+}, Ca^{++} and Co^{++}. The enzymes were markedly inhibited by Zn^{++} and Cu^{++}. 10^{-2}M Hg^{++} completely inactivated all the three proteases.

Substrate Specificity (Protease and Peptidase Activity):

All the proteases were found to hydrolyse BAPA and BAEE but not BTEE. The proteolytic and peptidase activities by each of the proteases were determined under identical conditions. The results are summarized in Table VIII. It can be seen that the protease I is most active against casein as well as BAPA. Though protease III was more active than protease II against casein, the situation was reverse for BAPA as substrate.

Effect of Various Substances:

To understand more about the nature of the enzymes, the effect of various substances on protease activity was studied (Table IX). Metal chelating agent, EDTA, did not show any inhibitory effect on the proteolytic activity of each proteases at

Figure - 23 : Effect of guanidine hydrochloride on the peptidase activity of trypsin and *S. litura* alkaline proteases.

5-8 µg enzyme protein in a total volume of 1.0 ml contained requisite amount of guanidine hydrochloride in 0.1M Tris buffer, pH 8.0, to final molarity as indicated. After incubation for 30 minutes at 35°C, the residual activity was determined against BAPA as described in the text.

Trypsin ●

Protease I ○

Protease II ▲

Protease III Δ

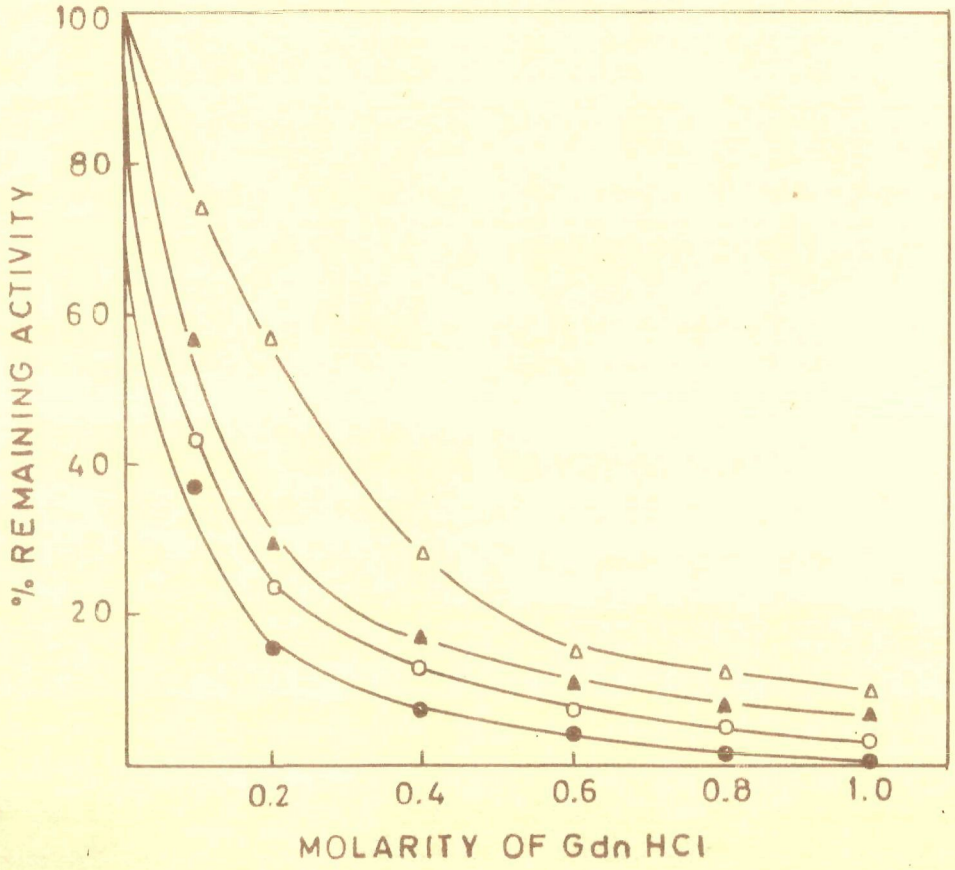

100
80
60
40
20
% REMAINING ACTIVITY
0.2
0.4
0.6
0.8
1.0
MOLARITY OF Gdn HCl

TABLE - VII

EFFECT OF VARIOUS METAL IONS ON THE PROTEOLYTIC ACTIVITY OF S. litura ALKALINE PROTEASES.

The reaction mixture in a total volume of 1 ml contained 30 umoles Tris buffer, pH 8.0, 5-8 µg enzyme protein and 10 mg casein. After incubation at 40°C for 15 minutes, the caseinolytic activity was determined as described in the text. 10 µmoles metal ions were added prior to the addition of casein and pre-incubated for 60 minutes at 35°C.

Additions	Relative Activity (%)		
	Protease I	Protease II	Protease III
None	100	100	100
Mg^{++}	108	100	100
K^{+}	100	92	100
Ca^{++}	107	100	95
Mn^{++}	103	92	96
Co^{++}	100	101	100
Hg^{++}	0	0	0
Cu^{++}	58	67	88
Zn^{++}	40	30	45

TABLE - VIII

ACTIVITY OF THREE ALKALINE PROTEASES OF *S. litura*.

Proteases	Specific activity against casein. (Units/mg)	Specific activity against BAPA. (Units/mg)
P_I	150	1.08
P_{II}	12	0.11
P_{III}	29	0.013

TABLE - IX

EFFECT OF VARIOUS SUBSTANCES ON THE PROTEOLYTIC ACTIVITY OF *S. litura* ALKALINE PROTEASES

The reaction mixture in a total volume of 1 ml contained 30 umoles Tris buffer, pH 8.0, 5-8 ug enzyme protein and 10 mg casein. After incubation at 40°C for 15 minutes the caseinolytic activity was determined as described in the text. Various substances, as indicated, were added prior to the addition of casein and pre-incubated for 30 minutes at 35°C.

Additions	Final Concentration	Relative Activity (%)		
		Protease I	Protease II	Protease III
None	-	100	100	100
EDTA	2×10^{-3} M	110	120	116
L-Cysteine	10^{-3} M	105	110	112
Monoiodoacetic Acid	10^{-2} M	111	95	116
L-Ethylmaleimide	10^{-2} M	106	106	110
β-Mercaptoethanol	2×10^{-3} M	100	105	96
PCMB	10^{-3} M	101	91	100
N-Bromosuccinimide	10^{-3} M	12	26	33
N-Bromosuccinimide	10^{-2} M	0	0	0

the concentration of 10^{-2}M . The activity of each proteases remained unaltered after the treatment with the thiol specific reagents, iodoacetic acid, N-ethylmaleimide, PCMB, β-mercapto-ethanol and cysteine hydrochloride. All the proteases were completely inhibited by 2 X 10^{-3}M N-bromosuccinimide.

Effect of Specific Inhibitors:

In order to determine the amino acid residues present at the active site of the proteases, the enzymes were incubated with various inhibitors for 30 minutes at 35°C. The residual activity was measured using BAPA as substrate. Table X shows the comparison of the inhibition characteristic of the S. litura proteases and bovine pancreatic trypsin. PMSF, which is known to react specially with functional serine residue, inhibited all the alkaline proteases of S. litura. Protease I and II were most susceptible to PMSF and protease III was as sensitive as trypsin. The specific inhibitor of trypsin, TLCK, showed pronounced effect on all the proteases and inhibition was similar to trypsin. The chymotrypsin specific inhibitor, TPCK, had no inhibitory effect on any of the enzymes. 10^{-5}M SBTI caused about 70% inhibition of each proteases in comparison to complete inhibition of trypsin. Each of the proteases were also inhibited by 10^{-4}M LBTI though the inhibition was only 30-50%. Ovomucoid only inhibited the protease I and II.

TABLE - X

EFFECT OF SPECIFIC INHIBITORS ON *S. litura* ALKALINE PROTEASES

The reaction mixture in a total volume of 1 ml contained 0.0010 - 0.0015 μmoles enzyme protein and the requisite amount of inhibitors in 0.1M Tris buffer, pH 8.0, to final molarity as indicated. After incubation for 30 minutes at 35°C, the residual activity was determined against BAPA as described in the text.

Additions	Concentration	Inhibition (%)			
		Trypsin	Protease I	Protease II	Protease III
PMSF	5×10^{-3} M	58	27	19	52
TLCK	4×10^{-3} M	96	97	90	87
TPCK	10^{-3} M	0	0	0	0
SBTI	10^{-5} M	100	73	72	69
LBTI	10^{-4} M	100	40	50	34
Ovomucoid	10^{-4} M	100	16	30	0

CHAPTER IV

D I S C U S S I O N

DISCUSSION

It is well known that proteins and carbohydrates are the chief constituents of insect food. Numerous attempts have been made to study the insect proteases in order to gain a better understanding of their properties and role in nutrition. Ishaaya et al. (1971) have reported the occurrence and preliminary properties of a protease with unusually alkaline pH optimum from the gut of Prodenia litura but no systematic attempts have been made to purify and characterize the enzyme.

It is evident from the changes in protease activity and protein concentration that the protein concentration appears to influence the protease activity. During day 7 to day 9 (Fig. 1), the larvae become more voracious feeder. This, presumably, accounts for the enhanced protein concentration in the gut. The protein concentration falls after day 9 as the larvae gradually become pupae and give up food consumption. Such changes in gut protease activity during development have also been demonstrated in larvae of Bombyx mori (Fujii and Kato, 1930; Matsumura and Oka, 1936). The protease activity of Galleria mallonella (Janda and Krieg, 1969) and Lygus disponsi (Hori, 1973) also varied markedly with the developmental stages having a sharp rise just before ecdysis.

Starvation profoundly affects the protease activity. There was a slight increase in protease activity during first 4 hours

of starvation which consistently declined on further starvation as also shown earlier with Bombyx mori (Fujii and Kato, 1930). At 4 hours, there was a very marked decrease in protein concentration of gut fluid, presumably, due to proteolysis which accounts for the marked increase in specific activity. Changes in protein concentration and protease activity during further starvation also suggest a relationship between them. It is of interest to mention here that feeding of the larvae with high protein diet causes a marked stimulation in protease activity (Ishaaya et al., 1971). Moreover, the observation that proteolytic activity of several insects decreased during starvation and increased again on refeeding (Dadd, 1956; House, 1965; Englemann, 1966, 1969; Janda and Krieg, 1969) suggests the influence of the protein on gut proteolytic activity.

Insect gut proteases usually have pH optima in the neutral and alkaline region (Gilmour, 1961; House, 1965) and the protease of S. litura appears to be no exception. As evident from the Fig. 3, the protease activity was maximum at pH 11.0, with a shoulder between pH 8 to 9, suggesting the presence of more than one protease. This is supported by the study of pH dependence of the partially purified enzyme which exhibited no shoulder between pH 8.0 and 9.0 (Zafeer et al., 1976).

As the optimum pH of the enzyme was 11.0, it was hoped that most of the proteins would be unfolded at this pH and would be degraded by the alkaline protease. Incubation of the enzyme,

therefore, at room temperature was expected to degrade the contaminating proteins and lead to extensive purification. As evident from Table I, incubation for 22 hours at 37°C lead to three-fold increase in specific activity but further increase in incubation time resulted in no further protein degradation and a slight decrease in specific activity was observed. The observed inactivation of the enzyme on dialysis at room temperature could be due to autolysis in cold failed to inactivate the enzyme (Table II). It is well known that autolysis of proteolytic enzyme is inhibited by autolysed product (Determann et al., 1969). In order to separate the degraded products, column chromatography was performed on Sephadex G-75 (Fig. 4). It is evident from the preliminary report (Zafeer et al., 1976) that the enzyme was not homogeneous indicating three markedly resolved bands on polyacrylamide gel electrophoresis. To have better knowledge about the nature of the protease, further work was done to purify the component proteases to homogeneity.

Three proteases (P_I - P_{III}) have been purified to homogeneity from the gut of S. litura. Insects having more than one proteases have also been purified by different authors (Applebaum et al., 1964; Giebel et al., 1971; Knecht et al., 1974; Ward, 1975). Proteolytic enzymes can be classified in one of the four groups (a) Serine proteases, (b) acid proteases, (c) thiol proteases, and (d) metal activated proteases (Hartley, 1960). The thiol specific reagents failed to substantially inhibit either of the proteases. The metal chelating agent EDTA

also had no effect. These proteases required no metal ions for their full activity. Ca^{++}, Co^{++}, Mg^{++} and Mn^{++} had almost no influence on the activity whereas the heavy metals like Zn^{++} and Cu^{++} caused a strong inhibition. However, Hg^{++} caused complete inhibition of all the three protease (Table VII). This inhibition by Hg^{++} is unsufficient evidence to class the enzymes as thiol proteases (Hartley, 1960) and certainly as shown in Table VII neither of the enzymes can be considered a metal activated enzyme. Proteases I - III have been classified as alkaline proteases on the basis of their pH optima in alkaline region. Protease I, II and III had maximal activity at pH 11.0, 10.5 and 9.0 respectively. Most of the insect alkaline proteases have pH optima around 8.0 - 9.0 (Kafatos et al., 1967; Hagenmaier, 1971; and Knecht et al., 1974). An alkaline protease from the midgut of Bombyx mori with pH optima at 11.0 has been reported by Eguchi and Iwamoto (1976). Alkaline proteases with 10 - 11 pH optima has been reported by several authors in bacterial (Boething, 1975; Peter and Leon, 1974; Lask and Blackburn, 1971) and fungal system (Iguchi and Yamamoto, 1955; Hayashi et al., 1970). With respect to temperature optima protease I resembled the trypsin-like protease of Vespa orientalis which also exhibited maximum activity at 60°C (Hagenmaier, 1971). Protease II was similar to procine trypsin which had maximum activity at 55°C (Travis and Liener, 1965). Like bovine trypsin, protease III had maximal activity at 50°C (Buck et al., 1962).

Like bovine trypsin protease I and II retained partial activity after 45 minutes at pH 2.4. However, unlike bovine trypsin,

protease III is rapidly inactivated at a pH range that is mildly acidic (pH 3 to 4). The thermal stability of protease I - III was very much similar to each other. The vertebrate and some invertebrate trypsins are markedly stabilized by Ca^{++} (Nord and Bier, 1953; Green, 1953) and activated by Ca^{++} (Sipos and Merkel, 1968). In contrast Ca^{++} had virtually no effect on stability and activity of the S. litura proteases.

All the proteases were inhibited by PMSF and TLCK, suggesting an essential role of a serine (Dixon et al., 1956) and a histidine residue (Shaw et al., 1965) respectively, in the catalytic function of these enzymes. The proteases have been referred to as "trypsin-like" on the basis of their ability to hydrolyse the synthetic substrates BAPA and BAEE. Inhibition by SBTI and LBTI also suggest the homology of S. litura proteases with vertebrate trypsins. Relatively ovomucoid had more inhibitory effect on protease II than I while protease III was not inhibited. With respect to ovomucoid inhibition protease III behaved like human trypsin (Buck et al., 1962). Trypsin-like protease from insect species which are inhibited by PMSF, TLCK, SBTI, LBTI and ovomucoid are reported from Apis mellifica (Giebel et al., 1971), Vespa orientalis (Hagenmaier, 1971) and Locusta migratoria (Knecht et al., 1974). N-Bromosuccinimide which causes the inactivation of chymotrypsin (Viswanatha and Lawson, 1961) and trypsin (Viswanatha et al., 1960) by destroying the tryptophan residue of the enzyme molecule, also strongly inhibited the enzymatic activity of S. litura proteases.

Protease I and II have molecular weights of 17,000 and 21,000 respectively by gel filtration which are similar to one of the tryptic enzymes from Apis mellifica (Geibel et al., 1971) having a molecular weight of about 20,000. A molecular weight of about 18,000 was observed for the tryptic enzyme of Locusta migratoria (Knecht et al., 1974). The molecular weight of a tryptic protease in the beetle Pterostichus melanorius found by gel filtration method was about 16,600 (Gooding and Huang, 1969). Protease III appears to be an exception of having molecular weight of 53,000 by gel filtration. The non-appearance of more than one band on SDS polyacrylamide gel electrophoresis of all the three proteases ruled out the possibility of native protein III to exist in dimeric or trimeric form.

The proteases of S. litura differ from vertebrate trypsins in the apparent absence of any of these enzymes in an inactive form. At one time it was thought that the lack of protease zymogens may be a general characteristic of all invertebrates (Neurath et al., 1967; Winter and Neurath, 1970) but Camacho et al. (1970) have subsequently reported an inactive trypsinogen in the starfish Dermasterias imbricata. The special trypsin like enzyme, cocoonase also exists as an inactive zymogen (Felsted et al., 1973) and zymogens for the trypsin-like enzymes in the lower vertebrates Squalus acanthias (Bradshaw et al., 1970), Protopterus aethiopicus (Reeck and Neurath, 1972) and Tineola bisselliella (Ward, 1975) have also been reported.

The present results clearly indicate close similarities between S. litura proteases and vertebrate trypsins. In particular, the results obtained with specific inhibitors PMSF and TLCK suggest the homology with vertebrate trypsins but definite evidence await detailed structural analysis. Since protease I - III are inhibited by PMSF and have pH optima between 9 to 11, these enzymes have the general characteristics of an alkaline protease (Matsubara, 1971).

Whether these alkaline proteases of S. litura represent the separate gene products or they are modifications of the same enzyme is a matter of speculation. The latter possibility is more likely as suggested by the absence of protease III few times or getting in only small quantity. A possible explanation of this may be the limited autolysis of protease III to get protease I and II. The results may be compared to metalo-proteinases of Chymobacterium lividum (Etherington et al., 1976).

The further proof of the possibility that protease I and II are derived from protease III is understood from our previous report (Zafeer et al., 1976). Incubation at 37°C for 22 hours and partial purification on Sephadex G-75 resulted one minor and one major active peak. This minor peak probably belonged to the same shoulder at pH 8-9 (Fig. 3) since the pH-activity profile of major peak resulted in the removal of this shoulder. Thus, this minor peak probably resembled the protease III and the major peak

to the protease I and II suggesting the derivation of protease I and II from protease III. Since the protease III was obtained only few times and in very small quantity we could not confirm this possibility by checking whether protease I and II are derived from protease III.

SUMMARY

The alkaline protease activity in the gut of Spodoptera litura was found to increase with the development of larvae and decreased with the onset of pupation. The pattern of changes in protein concentration in the gut was parallel to the protease activity. During day 7 to day 9, the larvae became more voracious feeder which presumably accounts for the enhanced protein concentration in the gut. The protein concentration falls after day 9 as the larvae gradually become pupae and give up food consumption.

The change in protease activity during starvation of the larvae showed an abrupt increase at 4 hours which declined consistently on further starvation. There was a slight decrease in protein concentration of gut content at 4 hours with a slower decline on further starvation.

Incubation of crude enzyme solution at 37 ± 1°C for 55 hours resulted in a sharp decrease in protein concentration until 22 hours without any further significant change until 55 hours. The enzyme activity remained almost unaltered till 22 hours but decreased subsequently till 55 hours. A 42% loss in enzyme activity was observed when the crude enzyme solution was dialysed at 37°C, whereas only 10% loss in activity was observed at 4°C.

To purify the alkaline proteases, the fifth instar larvae of Spodoptera litura were dissected and their intestines collected in an ice cold beaker. The contents were squashed out by means of a glass rod in 0.1M Tris buffer, pH 8.0, to get the crude enzyme solution. Since the crude extract contains dark brown pigments and phenols, it was removed by acetone fractionation. The acetone fraction was passed through Sephadex G-75 followed by exchange chromatography on DEAE-Sephadex A-50. The alkaline proteases appeared in three well defined peaks which were homogeneous as judged by plyacrylamide gel electrophoresis. The percentage of recovery for protease I - III were 18.7, 19.4 and 13.6 with 9, 7 and 8 fold purification. Protease I - III exhibited molecular weight values of 17000, 21000 and 53000 by gel filtration on Sephadex G-200 and 18000, 23000 and 50000 by sodium dodecylsulfate polyacrylamide gel electrophoresis, respectively. The non-appearance of more than one band on SDS polyacrylamide gel electrophoresis of all the three proteases rules out the possibility of native protein III to exist in dimeric or trimeric form. The Stoke's radii for protease I - III were calculated as 1.89 nm, 2.16 nm and 3.08 nm respectively. The values of diffusion constant for the three proteases were respectively 11.79 X 10^{-2} cm^2/Sec, 10.32 X 10^{-7} cm^2/ Sec and 7.24 X 10^{-7} cm^2/Sec. These observations suggested that protease I and II are more compact and globular than protease III.

The optimum pH for the crude extract was 11.0, with a shoulder between pH 8.0 and 9.0. Optimal pH for protease I - III

using casein as substrate were 11.0, 10.5 and 9.0 respectively. The temperature optima for protease I - III obtained by 20 minutes reaction at pH 8.0 were 60°C, 55°C and 50°C respectively. The effect of pH on the stability of enzyme showed that protease I and II are relatively more stable than protease III at extremes of acidic or alkaline pH. There was no remarkable difference in terms of thermal inactivation of all the proteases. The inactivation began at 45°C, and at 60°C, almost all the proteases lost fully activity. Lineweaver-Burk plots were obtained with each of the proteases using casein as substrate. The Km values obtained were 5.7×10^{-6} M, 2.9×10^{-6} M and 2.1×10^{-6} M respectively for protease I, II, and III. All the proteases hydrolysed BAPA, BAEE but not BTEE suggesting the trypsin-like nature. Protease I is most active against casein as well as BAPA as a substrate. Though, protease III was more active than protease II against casein, the situation was reverse for BAPA as substrate.

In an attempt to understand about the nature of the enzymes, the effect of various inhibitors on protease activity was studied. Metal chelating agent, EDTA, did not show any inhibitory effect on the proteolytic activity of each enzyme. The activity of each proteases remained unaltered after the treatment with the thiol specific reagents, iodoacetic acid, N-ethylmaleimide, PCMB, β-mercaptoethanol and cysteine hydrochloride. Like trypsin and chymotrypsin all the proteases were completely inhibited by N-bromosuccinimide. PMSF which is known

to react specially with functional serine residue, inhibited all the proteases. TLCK was inhibitory to all the proteases indicating the participation of histidine in the active site. The chymotrypsin specific inhibitor, TPCK had no inhibitory effect on any of the enzymes. Each of the proteases were inhibited by SBTI and LBTI but ovomucoid only inhibited the protease I and II.

These proteases required no metal ions for their full activity. Ca^{++}, Co^{++}, Mg^{++} and Mn^{++} had almost no influence on the activity whereas the heavy metal ions like Zn^{++} and Cu^{++} caused strong inhibition. However, Hg^{++} caused complete inhibition of all the three proteases.

No evidence could be found for the existence of any of these proteases as inactive precursors.

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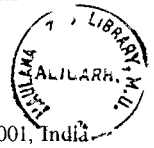
ALKALINE PROTEASE IN THE LARVAE OF THE ARMY WORM, *SPODOPTERA LITURA*



ZAFEER AHMAD, M SALEEMUDDIN*, and MAJID SIDDIQI
Biochemistry Division, Department of Chemistry, Aligarh Muslim University, Aligarh 202001, India

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Abstract—The alkaline protease activity in the gut of *Spodoptera litura* was found to increase with the development of larvae and decreased with the onset of pupation Fasting of the 5th instar larvae caused a slight increase in protease activity at 4 hr, which declined consistently on further starvation The optimum pH for the gut protease was 11 0, with a shoulder between pH 8 0 and 9 0 The protease was inactivated upon dialysis of the crude enzyme solution at room temperature but not at 4°C Incubation of the crude enzyme solution at pH 11 0 and 37°C for 22 hr resulted in a three-fold rise in specific activity of the alkaline protease which declined on further incubation The three-fold purified preparation, obtained by incubation of the crude enzyme solution, was passed through Sephadex G-75 to give a seven-fold purification and 70% yield The preparation was not completely homogeneous and showed three clearly separable protein bands by polyacrylamide gel electrophoresis The partially purified protease exhibited no shoulder between pH 8 to 9, like the crude preparation

INTRODUCTION

ISHAAYA *et al* (1971) have reported the occurrence and preliminary properties of a protease with an unusually alkaline pH optimum in the larvae of *Spodoptera littoralis* Boisd (= *Prodenia litura F*) However, no systematic attempts have so far been made to purify and characterize the enzyme The present work describes the changes in protease activity during development of the larvae of *S litura* and effect of fasting on proteolytic activity Attempts have also been made to purify and study some properties of the enzyme

MATERIALS AND METHODS

Sephadex G-75 was purchased from Pharmacia Fine Chemicals (Sweden) Vitamin-free casein was a product of Difco Laboratories (U S A) Haemoglogin was isolated from buffalo blood according to the method of DRABKIN (1946) The reagents used in polyacrylamide gel electrophoresis were N, N, N′, N′, tetra methyl ethylenediamine (BDH, UK), riboflavin (E Merck, Germany), Amido Schwartz (E Merck, Germany) and ammonium persulfate (PPH, Poland)

All other chemicals were of reagent grade and glass double distilled water was used throughout these studies

Rearing method

Soon after hatching, the larvae were bred at room temperature (30 ± 2°C) in a glass jar having moistened sand at the bottom They were fed daily with castor leaves Only fifth instar larvae, unless otherwise mentioned, were used

* Present address Goethe Institut, 78 Freiburg, Wilhelmstr 17, West Germany

Preparation of larval enzyme solution

After dissection of larvae, intestines were collected in an ice cold breaker Their contents were squashed out by means of a glass rod in 0 1 M Glycine NaOH buffer, pH 11 0 The mixture was centrifuged in the cold at 4000 rev/min for 20 min, and the supernatant used as enzyme solution

Determination of proteolytic activity

Protease activity was determined by the casein digestion method of KUNITZ (1947) with certain modifications The reaction mixture, in a total volume of 1 ml, consisting 40 μmole phosphate or glycine–NaOH buffer and 20 to 40 μg enzyme protein was pre-incubated for 5 min at 40°C 0 5 ml of 2% casein dissolved in 0 1 M buffer of desired pH was added and reincubated at 40°C for 15 min After digestion, intact casein was precipitated with 0 5 ml of 20% trichloroacetic (TCA) and removed by centrifugation The concentration of digested protein in the supernatant was determined by Folin reagent (LOWRY *et al*, 1951) in a Bausch & Lomb Spectronic 20 colorimeter at 660 nm Appropriate blanks were used in all experiments Under the experimental conditions, the enzyme activity was expressed as μg of tyrosine formed per min

The protein was estimated by the method of LOWRY *et al* (1951) The interfering phenolic substances present in the crude enzyme solution were removed by the method of KHANNA *et al* (1969) before using Lowry method

Polyacrylamide gel electrophoresis was performed at pH 8 2 as described by DAVIS (1964)

RESULTS

Protease activity during development

The change in proteolytic activity at different larval ages after hatching from the eggs is shown in Fig 1

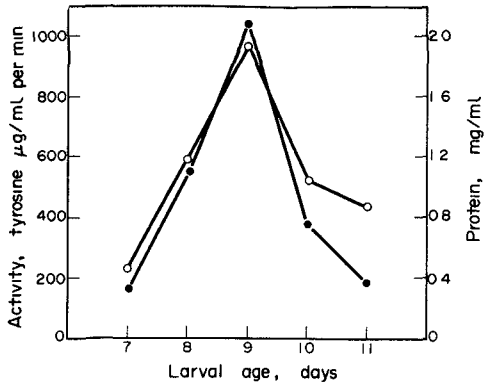

Fig 1 Protease activity during development of larvae of *S litura* ●——●, Protease activity, O——O, protein concentration

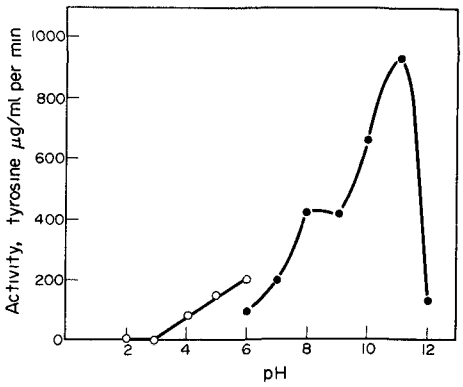

Fig 3 Effect of pH on the protease activity of crude enzyme solution Activity was measured from pH 2 0 to pH 12 0 in 0 1 M phosphate buffer O——O, Hydrolysis of acid denatured haemoglobin, ●——●, hydrolysis of casein

Maximum activity was observed on the 9th day which declined subsequently till day 11 The pattern of changes in protein concentration in the gut was parallel to the protease activity However, at 10 and 11 days, the decrease in proteolytic activity was slightly more in comparison to the decrease in protein concentration

Protease activity during starvation

The change in protease activity during starvation is shown in Fig 2 There was an abrupt increase in enzyme activity at 4 hr of starvation, after which a continuous decrease in the activity was observed till 24 hr The protein concentration of gut contents was found to decrease rapidly up to 4 hr of starvation followed by a slower decline until 24 hr Thus, the specific activity was increased two-fold at 4 hr and subsequently declined on further starvation

Effect of pH on protease activity of crude enzyme solution

The pH-activity curve is presented in Fig 3 Since it was difficult to prepare casein solution below pH 6 0, or acid denatured haemoglobin solution above pH 6 0, haemoglobin was used as a substrate from pH 2 0 to 6 0 and casein was used between pH 6 0 and pH 12 0 No enzyme activity was observed below pH 4 0 The activity of the enzyme increased until pH 11 0, with a shoulder at pH 8 0, and declined sharply above pH 11 0

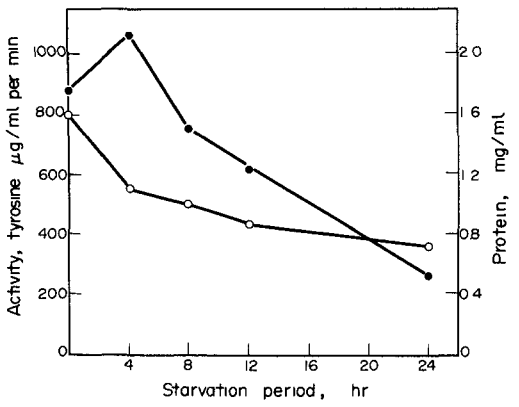

Fig 2 Protease activity during starvation period of 5th instar larvae ●——●, Protease activity, O——O, protein concentration

Changes in protease activity during incubation of crude enzyme solution

Crude enzyme solution was incubated at 37 ± 1°C for 55 hr and the specific activity was determined at different intervals The result is represented in Table 1 There was a sharp decrease in protein concentration until 22 hr of incubation without any significant change until 55 hr The enzyme activity remained almost unaltered till 22 hr but subsequently a loss in activity was observed and by 55 hr, 37% of the activity was lost Thus, incubation of the enzyme solution for 22 hr resulted in a three-fold increase in the specific activity which, however, declined on further incubation

Effect of dialysis on protease activity

Crude enzyme solution was dialysed for 24 hr against 0 1 M Glycine–NaOH buffer, pH 11 0, at 4°C

Table 1 Protease activity during incubation of crude enzyme solution at 37°C

Incubation periods (hr)	Protein (mg/ml)	Activity (tyrosine µg/ml per min)	Specific activity (tyrosine µg/mg protein)
0	3 67	937	225
8	1 35	851	630
22	1 15	851	740
30	1 15	741	644
55	1 12	592	528

Table 2 Protease activity of crude enzyme solution after dialysis for 24 hr

Preparation	Activity (tyrosine μg/ml per min)	% Inactivation
Undialysed	552	0
Dialysed at 4°C	497	10
Dialysed at 37°C	321	42

and 37°C As shown in Table 2, there was a 42% loss in enzyme activity when the enzyme solution was dialysed at 37°C, whereas only 10% loss in activity was observed at 4°C In a separate experiment, it was observed that the enzyme activity was completely lost after 40 hr dialysis at 37°C It is of interest to mention here that incubation of the enzyme solution for about 24 hr at 37°C caused very little inactivation

Purification of protease

The crude enzyme solution was incubated for 22 hr at 37°C To remove the pigments and phenols, the enzyme was precipitated by the addition of a three-fold volume of chilled acetone The precipitate was dissolved in 0 05 M Tris buffer, pH 8 0, and chromatographed through a column (2 0 × 35 cm) of Sephadex G-75 which had been equilibrated with the same buffer The flow rate was adjusted to 20 ml/hr and 3 ml fractions were collected The chromatographic pattern for this gel filtration is shown in Fig 4 Though protease activity was eluted as two peaks, majority of the activity was associated with the second The yield and degree of purity of the enzyme at each step of purification is summarized in Table 3

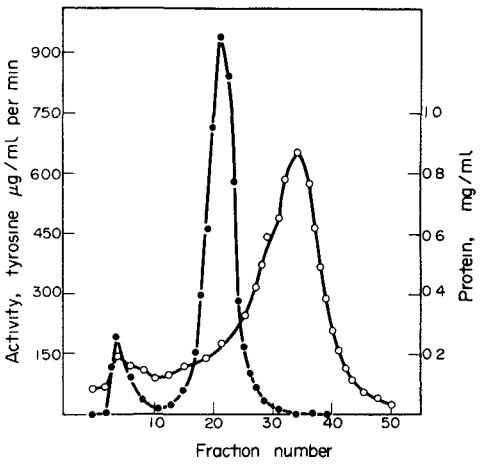

Fig 4 Gel filtration of the protease through Sephadex G-75 The column (2 0 × 35 cm) was equilibrated with 0 05 M Tris buffer, pH 8 0 3 ml fractions were collected at a flow rate of 20 ml/hr ●——●, Protease activity, ○——○, protein concentration

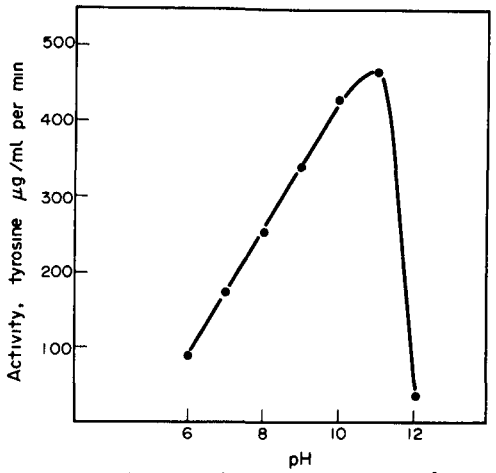

Fig 5 Effect of pH on the protease activity of partially purified enzyme Activity was measured from pH 6 0 to pH 12 0 in 0 1 M phosphate buffer Casein was used as a substrate

Polyacrylamide gel electrophoresis

Active fractions of the second peak were pooled and concentrated The pooled fractions were kept in a dialysis bag and placed in solid sucrose for concentration Polyacrylamide gel electrophoresis indicated three sharp and well separated bands Thus, the enzyme preparation was not completely homogeneous

Effect of pH on protease activity of partially purified enzyme

The pH–activity curve for crude enzyme solution suggests the presence of at least two proteases (Fig

Table 3 Purification of the protease

Purification step	Total protein (mg)	Total activity (tyrosine μg)	Specific activity (tyrosine μg/mg protein)	Purification	Recovery
Crude extract	117	20,280	173	1	100
Incubation at 37°C for 22 hr	38 25	19,968	522	3	98
2nd peak from Sephadex G-75	9 00	14,222	1580	7	70

3) However, unlike crude enzyme solution, the partially purified preparation exhibited no shoulder at PH 8 0 (Fig 5) It is likely that a proteinase having pH optimum around 8 0 has been removed The sharp decline in the activity of the purified preparation, after pH 11 0, was comparable to the crude enzyme solution

DISCUSSION

It is evident from the changes in protease activity and protein concentration that the protein concentration appears to influence the protease activity During day 7 to day 9 (Fig 1), the larvae become more voracious feeder This, presumably, accounts for the enhanced protein concentration in the gut The protein concentration falls after day 9 as the larvae gradually become pupae and give up food consumption Such changes in gut protease activity during development have also been demonstrated in larvae of *Bombyx mori* (FUJII and KATO, 1930, MATSUMURA and OKA, 1936) The protease activity of *Galleria mallonella* (JANDA and KRIEG, 1969) and *Lygus disponsi* (HORI, 1973) also varied markedly with the developmental stages having a sharp rise just before ecdysis

Starvation profoundly affects the protease activity There was a slight increase in protease activity during first 4 hr of starvation which consistently declined on further starvation as also shown earlier with *Bombyx mori* (FUJII and KATO, 1930) At 4 hr, there was a very marked decrease in protein concentration of gut fluid, presumably, due to proteolysis which accounts for the marked increase in specific activity Changes in protein concentration and protease activity during further starvation also suggest a relationship between them It is of interest to mention here that feeding of the larvae with high protein diet causes a marked stimulation in protease activity (ISHAAYA *et al*, 1971) Moreover, the observation that proteolytic activity of several insects decreases during starvation and increases again on refeeding (DADD, 1956, HOUSE, 1965, ENGELMANN, 1966, 1969, JANDA and KRIEG, 1969) also suggests the influence of the protein on gut proteolytic activity

Insect gut proteases usually have pH optima in the neutral and alkaline region (GILMOUR, 1961, HOUSE, 1965) and the protease of *S litura* appears to be no exception As evident from the Fig 3, the protease activity was maximum at pH 11 0, with a shoulder between pH 8 to 9, suggesting the presence of more than one protease This is supported by the study of pH dependence of the partially purified enzyme which exhibited no shoulder between pH 8 0 and 9 0

As the optimum pH of the enzyme was 11 0, it was hoped that most of the proteins would be unfolded at this pH and would be degraded by the alkaline protease Incubation of the enzyme, therefore at room temperature was expected to degrade the contaminating proteins and lead to extensive purification As evident from Table 1, incubation for 22 hr at 37°C lead to three-fold increase in specific activity but further increase in incubation time resulted in no further protein degradation and a slight decrease in specific activity was observed The observed inactivation of the enzyme on dialysis at room temperature could be due to autolysis as dialysis in cold failed to inactivate the enzyme It is well known that autolysis of proteolytic enzyme is inhibited by autolysed product (DETERMANN *et al*, 1969) In order to separate the degraded products, column chromatography was performed on Sephadex G-75 (Fig 4) A sevenfold purification was observed from the major peak eluted from G-75 Polyacrylamide gel electrophoresis indicated 3 markedly resolved bands indicating the heterogeneity of the preparation

It is evident from the present work that the alkaline protease is not homogeneous and thus no further studies were performed To have better knowledge about the nature of the protease attempts will be made to purify the enzyme to homogeneity

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